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BRAIN DYNAMICS OF PERSISTENT DEVELOPMENTAL STUTTERING: A MULTIMODAL NEUROPHYSIOLOGICAL PERSPECTIVE

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Abstract

Speech fluency in people with developmental stuttering (DS) is frequently interrupted by involuntary repetitions and prolongations of syllables, words, and sounds and/or by recurrent hesitations and pauses. Usually, DS occurs for the first time in early childhood and often remits spontaneously during puberty. However, in many cases it persists in adulthood. The combination of different therapeutic approaches may result in decent stuttering severity improvements. Nevertheless, a decisive rehabilitative solution, especially for adults, is not yet available.

In the last decades, the brain functioning of individuals with DS has been extensively investigated through numerous neuroimaging and neurophysiological studies. A series of "neural markers" suggests that stuttering may be the result of deficient neural dynamics in brain networks that support motor behavior, speech processing, and cognition. Dysfunctional activity within brain structures associated with motor planning, execution, and control is evident also in the absence of speech tasks, thus suggesting that dysfluencies may be only the overt symptom of a more general motor disorder. However, despite the crucial role of the motor system in DS, only a basic knowledge of its neural correlates is still available.

By using a multimodal non-invasive neurophysiological approach (e.g. TMS, EEG, TMS/EEG co-registration, and MEG, also in combination with source imaging and structural MRI information), this dissertation intends to fill important research gaps in stuttering, such as those related to i) the dynamics of neural networks in DS, ii) the muscular interplay during movement execution, iii) the effects of "social" stress on dysfluencies and speech motor programs.

The results highlight the critical role of the supplementary motor area (SMA) in the disturbance, and show that the often reported left hemisphere speech/motor under-activations may be counteracted by a mechanism in which cortical structures of the right hemisphere may react, in a "delayed" attempt of compensation. They also shed light on how external sensorial cues may help in improving the regulation of neural motor commands, proposing a mechanism by which the neural system may favor the preparation and control of motor sequences. Finally, they show that social and cognitive stress may negatively modulate the activity of the SMA "complex" and related regions, such as the anterior cingulate cortex, further contributing to perturb the neural exchange between speech and motor networks that precedes speech production.

As a consequence, the multimodal non-invasive neurophysiological approach adopted in the present dissertation provides further contributions to the current understanding of the neural

substrates that underlie the pathophysiological mechanisms of DS. Results may be useful to improve the available rehabilitation strategies, as well as to drive the realization of new and more tailored evidence-based interventions for this under-evaluated disturbance.

Riassunto

Nelle persone con balbuzie evolutiva, la normale fluenza del linguaggio è spesso interrotta da ripetizioni e prolungamenti involontari di sillabe, parole e suoni e/o da esitazioni e pause ricorrenti. La balbuzie evolutiva è un disturbo che compare nella prima infanzia e spesso scompare spontaneamente durante l'adolescenza tuttavia, in un certo numero di persone persiste nell'età adulta. Sebbene numerosi approcci terapeutici siano attualmente impiegati per il trattamento di questo disturbo, una terapia completamente efficace e risolutiva, specialmente per gli adulti, non è ancora disponibile.

Negli ultimi decenni il sistema nervoso centrale di bambini e adulti con balbuzie evolutiva è stato ampiamente studiato in numerosi studi neurofisiologici e di neuroimaging. Tali studi hanno permesso l'identificazione di una serie di "marker neurali" del disturbo, i quali suggeriscono che la balbuzie possa essere il risultato di anomale dinamiche neurali tra network cerebrali coinvolti nella realizzazione di compiti motori, nell'elaborazione del linguaggio e nei processi cognitivi. In particolare, l'evidenza di una anomala attività delle strutture cerebrali coinvolte nella pianificazione, esecuzione e controllo dei movimenti, anche non in concomitanza con compiti di produzione linguistica, ha permesso di ipotizzare che le disfluenze possano essere solamente il sintomo manifesto di un disturbo motorio più generale. Sebbene sia evidente un ruolo cruciale del sistema motorio nella balbuzie, solamente una conoscenza di base dei suoi correlati neurali è attualmente disponibile.

Attraverso un approccio neurofisiologico non invasivo e multimodale (TMS, EEG, co-registrazione TMS/EEG e MEG, in combinazione con MRI) questa tesi si prefigge di accrescere la conoscenza del substrato neurale che sottende il meccanismo fisiopatologico della balbuzie evolutiva in età adulta. Lo scopo degli studi presentati in questa tesi è quello di comprendere le dinamiche neurali della balbuzie, di studiare l'influenza reciproca tra diversi distretti muscolari durante l'esecuzione dei movimenti (anche in quelli non collegati al linguaggio) e di comprendere gli effetti dello stress sociale sulla manifestazione delle disfluenze e, perciò, sui meccanismi di preparazione motoria del linguaggio.

I risultati evidenziano il ruolo cruciale dell'area supplementare motoria nei meccanismi fisiopatologici della balbuzie e dimostrano come le ridotte attivazioni delle aree motorie e di quelle deputate al controllo del linguaggio nell'emisfero sinistro vengano contrastate da una anomala reazione delle strutture corticali dell'emisfero destro, in un tentativo "tardivo" di compensazione. Evidenziano inoltre come la presentazione di segnali sensoriali esterni possa facilitare la regolazione dei comandi motori, proponendo l'esistenza di un meccanismo

attraverso il quale il sistema nervoso può favorire la preparazione ed il controllo delle sequenze motorie. Infine, evidenziano come, nelle persone con balbuzie, lo stress sociale e cognitivo possa modulare negativamente l'attività dell'area supplementare motoria (e delle regioni collegate, come per esempio l'area del cingolo anteriore) interferendo ulteriormente con l'attività neurale dei network motori e del linguaggio che precede la produzione linguistica.

L'approccio neurofisiologico multimodale adottato nella presente tesi permette di accrescere la comprensione del substrato neurale che sottende i meccanismi fisiopatologici della balbuzie evolutiva persistente in età adulta. I risultati potranno contribuire al miglioramento delle terapie attualmente disponibili, nonché favorire la realizzazione di nuove e più mirate strategie riabilitative per questo disturbo troppo spesso sottovalutato.

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1 Introduction

Spoken communication is a form of verbal communication in which concepts and thoughts are translated into articulated sounds, combined to form words, and then encoded and interpreted in the speech perception process of the listeners. Brain regions involved in these mechanisms include those that are commonly associated with acoustic and phonological processing of speech and those that are usually associated with motor planning, execution, and control (Tourville and Guenther, 2011). Speech production represents one of the distinguishing features of humans and it is probably the most complex motor act that people perform every day. Indeed, the correct articulation of speech organs requires the selection of the proper motor sequences, the appropriate spatial and temporal activation of hundreds of muscles belonging to various body districts, and thus a complex exchange between different neural networks (Neef *et al*., 2015a; Chang *et al.*, 2019).

As in any other motor act, disruptions in speech production mechanisms may occur at different levels however, due to the complex nature of this phenomenon, even small perturbations may have a detrimental outcome on the final fluent speech generation and therefore on the effective communication process. This is what happens in stuttering - the normal rhythmic flow of speech is frequently interrupted by involuntary repetitions and prolongations of syllables, words, and sounds and/or by recurrent hesitations and pauses (World Health Organization – International Classification of Diseases-10). Dysfluent speech is the core feature of stuttering and it is also often associated with secondary concomitants that may include facial tension and abnormal movements of various body districts, not limited only to facial muscles (Craig-McQuaide *et al.*, 2014). Stuttering is common across all ethnicities and cultures, with no evident link to socioeconomic status, and can affect people of all genders and ages (Yairi and Ambrose, 2013). Everyone can experience some non-pathological dysfluencies during his daily life without a negative outcome on his verbal communication process. However, in people who stutter, dysfluencies specifically and chronically inhibit the ability to produce a fluent speech (Chang *et al*., 2019). Therefore, stuttering negatively influences the speech communication attitude of affected individuals and frequently impacts their quality of life and normal daily activities with a detrimental outcome on their emotional stability and mental health status (Craig *et al*., 2009). In this light, children and adults with stuttering usually show lower social interaction capacities, lower self-esteem (Iverach and Rapee,

2014), educational and occupational disadvantages (Klein and Hood, 2004), self-imposed isolation and elevated levels of trait, and social anxiety (Craig and Tran, 2014; Iverach and Rapee, 2014). Even though in some cases stuttering appears for the first time, especially in adulthood, as a result of acquired neurological disturbances (Lundgren *et al.*, 2010, Dinoto *et al*., 2018), brain damages (Grant *et al*., 1999), drug consumption (Brady, 1998), or psychological/emotional trauma (van Borsel and Taillieu, 2001) in the majority of cases it emerges with no apparent reasons in early childhood as a developmental disorder (Yairi and Ambrose, 2013). This form, known as "*developmental stuttering"* (DS), mainly affects male preschoolers and often remits spontaneously or following specific speech/behavioral therapies during adolescence however, it persists lifelong into a relatively small group of individuals (Yairi and Ambrose, 2013). DS is a very complex and highly heterogeneous condition in terms of symptoms severity: the individual manifestation of stuttering behavior may fluctuate during the life (Neef *et al*., 2015a) and across different social and emotionally-demanding contexts (Alm, 2014).

Past decades have seen the rapid development of several theories to explain the origin of DS. As well, a considerable amount of literature has disclosed the presence of diffuse structural and functional abnormalities in the neural system of children and adults with DS (Etchell *et al*., 2018). Taken together, all these studies, highlight the existence of a neurological basis of stuttering, however within the scientific community there is not a consensus on which anomalies play a causal role in DS and which are a consequence of compensatory strategies developed by the neural system to overcome the symptoms.

Numerous therapeutic approaches ranging from speech focused interventions to behavioral and psychological therapies have been employed in the management of stuttering (Brignell *et al.,* 2020). The combination of different approaches may result in decent improvements of stuttering severity however, a decisive rehabilitative solution, especially for adults, is not yet available (Connery *et al.,* 2021). No medication is officially approved for the treatment of stuttering, although dopamine blocking drugs (Murray *et al*., 1977; Maguire *et al*., 2000; 2004; Tavano *et al*., 2011) and other agents (Busan *et al*., 2009) have shown some efficacy in enhancing fluency and improving stuttering-associated symptoms. Even so, their employment is limited due to the high impact of side effects (Maguire *et al*., 2020). Recently, non-invasive brain stimulation approaches on different neural targets have produced some promising results (Chesters *et al*., 2018; Mejías and Prieto, 2019), yet the application of these techniques for the management of stuttering in clinical practice is a far-off prospect.

In recent years, significant analysis and discussions on the pathophysiological mechanisms underlying DS have been proposed by prominent authors (Alm, 2004a; Civier *et al*., 2010; 2013; Neef *et al*., 2015a; Chang *et al*., 2019; Busan, 2020; Chang and Guenther, 2020) nevertheless, the exact mechanism of DS onset and persistence, and its exact etiology are still largely elusive.

The following part of this dissertation moves on to describe in greater detail the features of DS and its functional and anatomical correlates in childhood and adulthood with the purpose to better specify the aims of the present work, as well as the successive studies descriptions and discussions. Unless otherwise stated the terms “stuttering” and “developmental stuttering” will be used interchangeably.

2 Developmental stuttering

Despite the considerable amount of literature published over the years, a complete, and satisfactory definition of stuttering is still not available (Onslow, 2020). Based on the objective definition provided by the World Health Organization in its "International Classification of Diseases-10" (WHO, 2015), dysfluencies represent the hallmark features of DS. In this regard, early observations (Johnson *et al.*, 1959), highlighted that eight types of dysfluencies are commonly evident during speech production in people who stutter:

1. Production of incomplete phrases (e.g. "I have to...with you")
2. Revisions (e.g. "I have to...I need to talk with you")
3. Interjections (e.g. "While I was – erm, um, uh – living in Italy")
4. Phrase Repetitions (e.g. "While I was, While I was living in Italy")
5. Whole-word repetitions (e.g. "While, while I was living in Italy")
6. Part-word repetitions (e.g. "S-s-stuttering")
7. Prolongations (e.g. "Sssstuttering")
8. Broken words (e.g. "I was liv-ing in Italy")

It is evident that none of the above-listed behaviors is exclusive to people who stutter but may sometimes be observed also in everybody's speech without failures in the verbal communication process. In this light, it has been proposed that speech symptoms of DS can be grouped in "less typical" which are evident both in normal speakers and in speakers with DS, and in "more typical", which occur more frequently in DS individuals, and may include whole and part word repetitions, prolongations and broken words (Jiang *et al., 2012*).

In people with DS, dysfluencies occur predominantly at syllable/word initial position or at the beginning of a phrase, in long words, in syntactically complex assertions as well as in meaningful words (Karniol 1995; Natke *et al.*, 2002). Dysfluencies occurrence typically decreases after the repeated aloud reading of the same written text (adaptation) and usually occurs in the same word or syllables in successive readings of the same passage (consistency) (Büchel and Sommer, 2004). Adaptation and consistency represent two of the distinctive features of persistent DS and are not evident in individuals with stuttering as a result of acquired brain damage (Lundgren *et al.*, 2010).

A series of associated symptoms that do not strictly involve speech organs usually accompany disfluencies (Craig-McQuaide *et al.*, 2014). These may include spasms, involuntary limb movements, abnormal gestures, and facial grimaces (Bloodstein and Ratner, 2008; Craig-McQuaide *et al.*, 2014). Voluntary movements, sometimes called "*starter movements*" or "*unblocking movements*" are frequently evident and typically represent a strategy to overcome the blocks and favor speech fluency (Riva-Posse *et al.*, 2008). Alterations in speech rate and vocal quality, severe blushing, and excessive perspiration can be also conspicuous and are often classified as "*physiological concomitants"* of DS (Bloodstein and Ratner, 2008; Craig-McQuaide *et al.*, 2014).

2.1 Epidemiology

It is estimated that almost 55 million people worldwide are affected from DS (Büchel and Sommer, 2004). In the majority of cases, DS occurs for the first time in early childhood between 2 and 9 years of age with a mean onset age of 33 months and with nearly 60% of onsets occurring in the third year of life (Yairi and Ambrose, 2005). The lifespan incidence of DS in the general population is about 8% with no clear differences among ethnicities (Yairi and Ambrose, 2013). Prevalence under the age of 6 ranges from 2.2% (Okalidou and Kampanaros, 2001) to 5.6% (McLeod and Harrison, 2009) on the other hand, in later periods of life (age sample 3-17 years) this parameter dramatically decreases (1.6% - Boyle *et al.*, 2011) and it is even lower (0.72%) when considering the entire age range (2-99 years- Craig *et al.*, 2002). As a matter of fact, many individuals recover either spontaneously or as a result of a specific speech/behavioral therapy (Yairi and Ambrose, 2013). Males to females ratio ranges from 0.66:1 in young children (age range 4-5 - Okalidou and Kampanaros, 2001) to 4.6:1 in later childhood and adolescence (age range 6-20 - van Borsel *et al*., 2006), with a ratio of 2.3:1 across all ages (age range 2-99 - Craig *et al.*, 2002). This suggests that the recovery rate may be higher in females than in males (Yairi and Ambrose, 2013). In terms of age of onset, there are no statistically significant differences between males and females and natural recovery occurs in 91% of cases (Yairi and Ambrose, 2013).

2.2 Etiology

Numerous theories on the etiology of developmental stuttering have been proposed since the ancient Greeks by scientists from different fields however, its neurobiological underpinnings are still obscure (Neef *et al*., 2015a).

Research investigating the factors associated with stuttering has focused on the possible relationship between speech apparatus abnormalities and/or the development of a psychological trauma. Based on empirical observations of stuttering disappearance during the so-called "*fluency inducting conditions*", such as choral speech or speaking to the pace of a metronome, many scientists have argued that DS may have its origin in the central nervous system at a speech motor planning level, rather than in the peripheral nervous system or in abnormalities of the vocal apparatus (Craig-McQuaide *et al*., 2014). This was further supported by the evidence of stuttering speech in normal speakers during the direct intraoperative electrical stimulation of brain regions such as the supplementary motor area (Penfield and Welch, 1951) the thalamus (Ojemann and Ward, 1971) thus suggesting the implication of cortico-subcortical circuits in the disturbance. Consequently, it has been proposed that stuttering may be the result of an aberrant interhemispheric relationship that can include the mistiming of nerve impulses to the speech muscle apparatus bilaterally (Travis, 1978).

Nowadays the key role of the neural system in DS is well documented. In the last decades, the rapid progress of advanced non-invasive brain-imaging methods has provided detailed descriptions of the neural system of children and adults who stutter (Etchell *et al.*, 2018), highlighting the existence of widespread brain anomalies especially in motor and speech-related areas both at rest and during concomitant behavioral tasks (see for example Sommer *et al.*, 2002; Watkins *et al*., 2008; Chang *et al*., 2008; Etchell *et al*., 2018).

However, a much-debated question is whether these abnormalities play a causal role in the disturbance or are the consequences of lifelong stuttering. Family and twin studies (Rautakoski *et al*., 2012; Yairi and Ambrose, 2013), as well as molecular biology investigations (Kang *et al*., 2010), suggest that many individuals may be genetically predisposed to develop stuttering during their life. Indeed, children with a first-degree relative who stutter are three times more at risk to develop stuttering symptoms (Maguire *et al.*, 2020).

Interestingly DS shares many features and comorbidities with other neurodevelopmental disorders such as Tourette's Syndrome (Abwender *et al.*, 1998) and Attention Deficit

Hyperactivity Disorder (Druker *et al*., 2019) Stuttering may arise also as the result of streptococcal infections (Alm, 2020) or after the exposition to abnormal prenatal testosterone levels (Dönmez *et al*., 2019)

Considering that genetic, epigenetic, and environmental factors interact across the life in the development of the structure of the central nervous system, a single-factor theory on the origin of developmental stuttering may not be exhaustive (Smith and Weber, 2017). Compatibly, DS has to be considered a multifactorial neurodevelopmental disorder, strongly characterized by abnormalities of the central nervous system.

2.3 Neural markers of developmental stuttering

It is now well established, thanks to numerous high-resolution brain imaging and neurophysiological studies, that the pathophysiology of DS is more complex than previously thought. Despite the lack of evidence for a single causal factor leading to DS, the systematic investigation of brain structure and function in hundreds of affected and non-affected individuals (Etchell *et al*., 2018) suggests the existence of various "neural markers" of the disturbance that may be involved at different levels in its onset, maintenance, and/or remission.

2.3.1 Abnormal asymmetries in functional activations

There is a large volume of published functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) studies highlighting anomalous brain activation patterns in individuals with DS, especially during speech and language tasks (Budde *et al*., 2014; Belyk *et al*., 2015). Despite divergent findings as the result of the different techniques employed and different subgroups of participants investigated, it is evident that a peculiar pattern of brain activations may accompany speech production in people with DS (Neef *et al*., 2015a). In this context, Fox and colleagues (Fox *et al*., 1996) conducted a seminal study that investigated regional activations and deactivations during reading tasks. These authors highlighted that in adults who stutter, speech production is associated with a general overactivation of different structures of the motor system including the supplementary motor area, the left globus pallidus, the cerebellum, and the insula. An anomalous right-lateralized activation of the primary motor cortex was also observed. Strong focal deactivations were evident in the left hemisphere between frontal and temporal cortices in areas implicated in verbal comprehension and verbal fluency. Further

investigations revealed the existence of other neurofunctional signatures of speech production in DS that included, for example, lower activations in the left hemisphere at the level of the ventral premotor cortex and planum temporale and bilaterally in the orofacial sensory-motor cortex (Watkins *et al*., 2008). Conversely, greater activations may be evident also in deeper structures such as the insula and in the midbrain at the level of the substantia nigra, extending to subthalamic, pedunculopontine, and red nuclei (Watkins *et al*., 2008). Functional state-dependent changes suggest that left and right functional anomalies play distinct and opposite roles in the disturbance (Braun *et al*., 1997). Speech production under fluency inducting conditions is associated with the normalization of the functional activity in the left hemisphere and with the persistence of the overactivations in the right hemispheric motor areas (Fox *et al*., 1996). In addition, fluent reading is accompanied by the abnormal and systematic activation of the right frontal operculum (RFO) whose magnitude negatively correlates with indexes of stuttering severity (Preibisch *et al*., 2003). In this light, right hemisphere overactivations seem not to be related to stuttering in a causal way but may rather represent a non-specific neuroplastic adaptation for the aberrant activity and signal transmission of the left hemisphere (Braun *et al*., 1997; Preibisch *et al*., 2003).

Collectively, functional anatomy investigation in individuals with DS outline a critical role for the hemispheric lateralization with a generally reduced activity of left hemisphere speech, motor, and auditory areas, and with the over-activity of right homologue fronto-temporal and rolandic areas (Neef *et al*., 2015a).

2.3.2 Abnormal regional gray matter brain volume

Since brain functioning cannot be separated from its structure, it is not surprising that functional and anatomical aberrancies may coexist in overlapping brain areas in people with DS. In this light, magnetic resonance-based morphological studies in children (Chang *et al*., 2008; Beal *et al*., 2013; Foundas *et al*., 2013; Garnett *et al*., 2018; Koenraads *et al*., 2019) and adults (Beal *et al*., 2007; Kell *et al*., 2009; Choo *et al*., 2011) who stutter have often reported abnormal brain volume especially in cerebral regions involved in speech processing and motor control (see Etchell *et al*; 2018 for a review).

Multiple publications have shown increased gray matter volumes in adults who stutter in the right superior temporal cortex (Beal *et al*., 2007; Song *et al*., 2007; Kikuchi *et al*., 2011) and especially in the right primary auditory cortex, as well as in the right cerebellum

(Beal *et al*., 2007). Increased grey matter volume may be evident also in the left temporal gyrus (Beal *et al*., 2007; Song *et al*., 2007), in the rostrum and in the midbody of the corpus callosum (Choo *et al*., 2011) as well as in the precentral and postcentral gyrus bilaterally (Song *et al*., 2007). Consistent with lower functional activations, a decrease in gray matter volume is evident in the left inferior frontal gyrus both in adults (Kell *et al*., 2009) and children (Chang *et al*., 2008; Beal *et al*., 2013; Koenraads *et al*., 2019) with DS. Relatively to fluent peers, children who stutter show decreased gray matter volumes also in the right inferior frontal gyrus (Chang *et al.*, 2008; Beal *et al*., 2013) in the planum temporale (bilaterally; Chang *et al*., 2008), in the left putamen (Beal *et al*., 2013) and in the right caudate nucleus (Foundas *et al*., 2013). Alterations in basal ganglia may be evident also in adults with DS in which a reduction of gray matter volume is present in the left caudate nucleus (Sowman *et al*., 2017) Interestingly, stuttering severity negatively correlates with gray matter volume of the left inferior frontal gyrus in adults (Kell *et al*., 2009) but not in children, in which a negative correlation is evident with the gray matter volume of the right pars triangularis and opercularis (Beal *et al*., 2013).

Significant differences may be evident also when comparing children who persisted in stuttering with those who recovered and fluent peers (Chang *et al*., 2008; Garnett *et al*., 2018; Koenraads *et al*., 2020). For example, children with persistent DS show less gray matter volume in the right cingulate gyrus while recovery is associated with bilaterally reduced volume in the cerebellum, in the medial temporal gyrus and in precentral gyri (Chang *et al*., 2008). Children with a history of stuttering may show reduced cortical thickness of the left frontotemporal and right parietal regions and reduced gray matter volumes in the supplementary motor area (Koenraads *et al*., 2019). Consistent with this evidence, cortical thickness is significantly decreased only in children with persistent DS in the left premotor and primary motor areas while recovery is associated with decreases in local gyrification indexes in the pre-supplementary motor area (Garnett *et al*., 2018).

2.3.3 White matter alterations

One of the most recurrent findings in DS brain structural imaging research is the alteration of white-matter microstructure evident in both children and adults with DS when compared with their fluent peers (Etchell *et al.*, 2018).

The first assessment of white matter integrity in DS, by means of diffusion tensor imaging (DTI), revealed reduced fractional anisotropy (FA) in the left hemisphere of adults with

DS at the level of the Rolandic operculum immediately below the sensory-motor representation of larynx, pharynx, and tongue (Sommer *et al*., 2002). This region is critical in the fluent speech production process since fiber tracts encompassing this area connect the sensorimotor representation of speech articulators with the left inferior frontal operculum and the left ventral pre-motor cortex. Similar findings are also reported in children with DS (Chang *et al*., 2008) thus suggesting that the neural disconnection below left primary motor cortex representations of oral articulators may represent a strong neural signature of the disturbance and may not be the mere result of the long term effects of stuttering. Further structural MRI studies revealed the existence of different and more diffuse white matter abnormalities in distributed networks. For example, children with DS showed white matter deficiencies along the left superior longitudinal fasciculus encompassing the inferior frontal gyrus, motor and pre-motor areas, superior temporal/middle temporal gyrus, and inferior parietal areas (Chang *et al*., 2015) as well as reduced white matter volume bilaterally in the forceps minor of the corpus callosum (Beal *et al*., 2013). Slightly reduced white matter integrity was also evident in the right hemisphere in tracts below the inferior frontal gyrus, the superior temporal/middle temporal gyrus, and the supramarginal gyrus (Chang *et al*., 2015). White-matter connectivity appeared to be reduced between the putamen and the supplementary motor area (Chang and Zhu, 2013). Conversely, adults with DS showed altered white-matter integrity in the left corticospinal tract, in the left middle frontal gyrus, in the left middle temporal gyrus and, bilaterally, in the arcuate fasciculus, in the ventral premotor cortex, and in the superior frontal gyrus (Watkins *et al*., 2008; Connally *et al*., 2014). Reduced white matter integrity is also found bilaterally in the frontal aslant tract (FAT) (Kronfeld-Duenias *et al*., 2016). The FAT is a bundle that connects the pars opercularis of the inferior frontal gyrus with the anterior supplementary motor area and pre-supplementary motor area (Catani *et al*., 2012). The FAT is critical in motor aspects of speech production and its anomalous white matter microstructure seems crucial in DS pathophysiology. Speech fluency negatively correlates with mean diffusivity within the left FAT in adults with DS (Kronfeld-Duenias *et al*., 2016) and the direct electrical stimulation of the FAT may result in transient stuttering in non-affected individuals (Kemerdere *et al*., 2016).

Widespread anatomical brain asymmetries are evident when considering the right hemisphere probably as a result of adaptive or maladaptive mechanisms. In this context, increased white matter volume is evident in the right precentral gyrus, close to facial motor

representations, in the right inferior frontal gyrus, and in the right superior temporal gyrus (Jäncke *et al*. 2004).

DNA sequencing in families with recurrent cases of stuttering revealed a possible genetic predisposition to develop white matter deficiencies but only in some individuals (Buchel and Watkins, 2010; Drayna and Kang, 2011; Raza *et al*., 2016). Stuttering is associated with missense mutations on the GNPTAB gene which encodes the α and β subunits of the *N*-acetylglucosamine-1-phosphate transferase (GlcNAc-1-phosphotransferase), on the GNPTG gene which indeed encodes the γ subunit and on the NAGPA gene that encodes N-acetylglucosamine-1-phosphodiester alpha-N-acetylglucosaminidase (Kang *et al*., 2010). These proteins are involved in the enzyme trafficking pathway to the lysosomes and in several intracellular processes that include also the biogenesis and maintenance of the myelin sheets (Buchel and Watkins, 2010). Their role in DS is further supported by more recent investigations. Mice engineered to carry the human GNPTAB mutation showed alterations in pup ultrasonic vocalizations that resemble that of humans and exhibited white matter abnormalities in the corpus callosum (Barnes *et al*., 2016; Han *et al.*, 2019).

Overall, the assessment of white matter structure and connectivity in people with DS highlights the existence of brain anomalies especially in structures involved in speech generation and in circuits crucial for motor planning, execution, and control. Although it remains unclear which of the above-described alterations favor the onset of the disturbance and which others are the consequence of plastic changes related to compensation, abnormal white matter connections in left speech/motor neuronal circuits may play a key role in DS.

2.3.4 Altered brain metabolism

Trait-dependent and state-dependent changes of glucose metabolism are evident in adults with DS in cortical areas and subcortical structures involved in speech production, emotion processing, and motor control (Wu *et al*., 1995; Wu *et al*., 1997). State-dependent glucose metabolic hypoactivity is evident in brain areas encompassing the left hemispheric language circuits including Broca's and Wernicke's areas as well as in the right superior frontal lobe, the left prefrontal cortex, the right cerebellum, the left deep frontal orbital cortex, and the bilateral posterior cingulate cortex during stuttering speech production (Wu *et al*., 1995). Induced fluency state normalize glucose metabolism in these areas but

it is also associated with excessive glucose uptake in the substantia nigra/ventral tegmental area, thus suggesting the existence of an increased compensatory neuronal firing in the midbrain (Wu *et al*., 1995). A trait-related region of glucose hypometabolism is instead evident in the basal ganglia at the level of the left caudate nucleus which is nearly 50% less active in DS individuals (when compared to normal speakers) and fails to normalize during induced fluency (Wu *et al*., 1995).

2.3.5 Excessive dopaminergic activity

A decrease in striatal and limbic glucose metabolic rates may be in part related to the excessive dopaminergic activity which is reported in adults with DS (Wu *et al*., 1997). When compared to fluent speakers, adults with DS show three times higher levels of the 6-Fluorodopa uptake, an index of presynaptic dopamine metabolism, in the left caudate tail and in the ventral medial prefrontal cortex which is a structure involved in the vocalization process in primates (Wu *et al*., 1997). Interestingly, the 6-Fluorodopa uptake levels are double the normal in the right auditory cortex and in limbic structures including the left amygdala, the left insular cortex, the left pulvinar, right hypothalamus, and the right deep orbital cortex (Wu *et al*., 1997).

Dopamine is a regulatory neurotransmitter and it is present in brain pathways involved in executive functions, action selection, and motor control (Graybiel, 2000). Excessive dopaminergic activity in the striatum may result in inefficient speech/motor program release and thus the abnormal regional dopamine metabolism may play a crucial role in DS (Alm, 2004a). Genetic susceptibility in developing stuttering is evident in some individuals who carry mutations in specific dopamine-related genes. This condition may result in lower dopamine receptor binding and thus in the exaggerated activity of the dopamine system in the striatum (Lan *et al.*, 2009). The key role of dopamine in DS pathophysiology is further supported by evidence from clinical pharmacological studies (Maguire *et al.*, 2020). In this context, fluency enhancement is often reported in some individuals after the administration of dopamine blocking drugs such as haloperidol (Murray *et al.*, 1977), which has proven to reverse abnormal right shift activations in speech structures (Wood *et al*., 1980), and risperidone (Maguire *et al*., 2000) which positively modulates brain activity in the striatum and in speech areas (Maguire *et al.*, 2020) and reduces tic-link motor behaviors of people who stutter (Tavano *et al*., 2011). Conversely, the administration of dopaminergic drugs such as L-Dopa may worsen

stuttering in affected individuals (Burd and Kerbeshain, 1991; Anderson *et al.*, 1999) or induce iatrogenic stuttering in patients with Parkinson's disease (Louis *et al.*, 2001).

2.3.6 Abnormal basal ganglia functioning

The multimodal investigation of the neural system of people with DS systematically highlighted the strong involvement of the basal ganglia in the disturbance (Wu *et al.*, 1995; Wu *et al.*, 1997; Alm, 2004a; Watkins *et al*., 2008; Ingham *et al*., 2012; Beal *et al*., 2013; Foundas *et al*., 2013; Sowman *et al*., 2017). Stuttering shares a series of features with many basal ganglia-related disorders such as Tourette's Syndrome (Abwender *et al.*, 1998) and focal dystonia (Kiziltan and Akalin, 1996) and its severity often correlates with abnormal basal ganglia activity (Giraud *et al*., 2008; Metzeger *et al*., 2018). Interestingly, stuttering may also re-emerge in patients with Parkinson's disease, and acquired neurogenic stuttering is often reported after basal ganglia lesions (Carluer *et al.*, 2000; Theys *et al*., 2013) especially in the left hemisphere (Alm, 2004a).

Basal ganglia represent the largest subcortical structures of the human forebrain and consist of a group of interconnected nuclei that comprise the striatum, the globus pallidus, the substantia nigra, and the subthalamic nucleus. The striatum is functionally divided into the caudate nucleus, putamen, and ventral striatum while the globus pallidus is divided into the globus pallidus external part (GPe), and the globus pallidus internal part (GPi) (Alexander 1994). Basal ganglia are the central hub of a broader structural and functional network known as cortico-basal-thalamo-cortical (CBTC) network that comprises also the thalamus and almost the entire cortical structures of the frontal lobe (Alexander 1994; Alm, 2004a). This network comprises a series of circuits involved in the full range of behaviors including motor control, cognitive processes, and limbic functions (Mink, 2018). The striatum receives excitatory glutamatergic projections from cortical neurons and modulates the activity of GPi through two different pathways that have the opposite effect on the final output. The activation of the direct pathway has an inhibitory effect on the GPi and thus results in the excitation of the cortical neurons. Conversely, the activation of the indirect pathway, which also includes the GPe and the subthalamic nucleus, exerts an excitatory effect on the neural activity of the GPi which in turn inhibits the cortex (DeLong, 2000).

When considering the CBTC motor loop, which has outputs to the primary motor cortex, supplementary motor cortex and premotor cortex (Alm, 2004a), the activation of the direct

pathway removes the tonic inhibition exerted by the thalamus on the motor generators of the final intended program and at the same time prevent the activation of motor pattern generators that may compete with the intended one (Mink, 2018). In the context of speech production, a failure in this mechanism may result in an anomalous integration of the excitatory and inhibitory signals to the muscles of the speech apparatus and thus hamper the correct activation of the final motor output and the transition from one syllable to the other. The activity of the two pathways is modulated by dopamine through the projections from the substantia nigra to the striatum. Dopamine has antithetic effects on the two pathways since excitatory D1 receptors are expressed mainly in the direct pathway and inhibitory D2 receptors are expressed mainly in the indirect one (Graybiel, 2000). In this light, the excessive dopaminergic activity reported in people with DS (Wu *et al.*, 1997) may interfere with the delicate balance of the direct and indirect pathways resulting in an incorrect release of the final motor output and/or in an insufficient suppression of competing motor programs (Alm, 2004a; Chang and Guenther, 2020).

2.4 Stuttering as a motor timing problem

The basal ganglia and the supplementary motor cortex are structures known to be crucial in providing the internal timing for the generation of motor sequences (Etchell *et al.*, 2014). In DS, dysfluencies occur mainly during the self-paced speech while conditions that involve an external pacing such as choral reading (Kalinowski and Saltuklaroglu, 2003) and speaking to a metronome (Brady, 1969) may transiently induce fluency in affected individuals. The activity of the caudate nucleus, globus pallidus and putamen is abnormally lower in people with DS during speaking but raises to normal levels when they perform metronome-timed speech (Toyomura *et al.*, 2011). However, the basal ganglia are not just underactive during concomitant speech production tasks but an enhanced activity of the putamen and globus pallidus may be also evident at rest (Watkins *et al.*, 2008; Ingham *et al.*, 2012). This suggests that the basal ganglia abnormalities in DS are not strictly speech-related but may also interfere with the correct execution of more generalized motor behaviors. In this context, dysfunctions are not restricted to the basal ganglia but are widespread within the CBTC network. For example, adults with DS exhibit abnormal functional connectivity in subcortical circuits from the putamen to the thalamus and in subcortical-cortical interactions between the thalamus and the pre-supplementary motor area (Lu *et al.*, 2010). Similar findings are evident in children with DS in which

reduced functional and structural connectivity is evident between the putamen and the supplementary motor area (Chang and Zhu, 2013). Structures within the CBTC network are crucial in fluent speech production since they are strictly connected with the speech motor regions and are strongly involved in providing the internal timing cue for speech initiation (Alm, 2004a; Etchell *et al*., 2014 and in the organization of syllabic motor programs (Lu *et al*., 2009; Chang and Guenther, 2020). In this light, abnormal basal ganglia activity along with dysfunctional thalamo-cortical connections to the supplementary motor cortex may be closely related to stuttering symptoms and thus have a significant role in its pathophysiology (Alm, 2004a; Lu *et al.*, 2010; Etchell *et al*., 2014; Chang and Guenther, 2020, Busan, 2020). That is to say, neurocomputational speech production models suggest that enhanced dopaminergic activity and abnormal basal ganglia functioning are associated with dysfluencies especially in the first part of the word while white matter impairments may affect the shift from one syllable to the next one (Civier *et al*., 2013).

2.4.1 The supplementary motor cortex – a central hub

The supplementary motor cortex (SMA complex) is one of the major outputs of the basal ganglia and is thus an integral part of the CBTC network. It is involved in the preparation of internally timed motor programs (Narayana *et al*., 2012; Etchell *et al*., 2014), in motor performance monitoring (Shima and Tanji, 2006), in the acquisition of new motor skills (Nachev *et al*., 2008), and also in speech and language processing (Hertrich *et al.*, 2016). The SMA complex is anatomically and functionally divided into a "proper supplementary motor area" (proper SMA), which lies rostral to the primary motor cortex representation of the foot, and into a "pre-supplementary motor area" (pre-SMA) which is located immediately anterior to the proper SMA and extends toward the prefrontal cortex (Kaas and Stepniewska, 2002; Nachev *et al*., 2008). The SMA complex controls various aspects of motor behavior and it is a central hub for speech production. Indeed, it is strongly connected not only with subcortical structures (Alm, 2004a) and primary motor cortices (Kaas and Stepniewska, 2002) but also with inferior frontal areas (through the FAT fascicle; Catani *et al*., 2012), with the cerebellum (Ruan *et al*., 2018), and with regions involved in cognitive aspects of the motor behavior and emotion processing (Nachev *et al*., 2008). Therefore its activity is crucial in different stages of speech generation. The rostral portion of the pre-SMA supports the lexical selection, the caudal part of the pre-

SMA is involved in linear sequence encoding and the control of articulatory motor output mainly relies on the activity of the proper SMA (Alario *et al.*, 2006). Interestingly, various speech disorders (Ziegler *et al*., 1997, Pai, 1999) including acquired neurogenic stuttering (Ackermann *et al*., 1996) may arise as the result of lesions in this area. Despite its key role in speech production and its involvement in stuttering-like acquired disorders (Abe *et al*., 1992) its structural and functional abnormalities have only been recently proposed as a further neural signature of DS (Neef *et al*., 2015a; Busan, 2020). In this regard, abnormal activation of the SMA is evident in DS at rest (Ingham *et al*., 2012), during dysfluent speech production (Brown *et al*., 2005), and during the generation of oro-laryngeal non-speech movements (Braun *et al*., 1997). Abnormal neurophysiological activity during speech motor preparation (Vanhoutte *et al*., 2016) may arise in relation to dysfunctional SMA activity and connectivity. Aberrant functional connectivity of the SMA may be evident at rest and during speech production in both children (Chang *et al*., 2015; 2018) and adults (Lu *et al*., 2010; 2012; 2016) with DS. For example, resting-state connectivity among the SMA complex, basal ganglia, and regions involved in the regulation of attention processes such as the posterior cingulate cortex (Chang *et al*., 2018) is reduced in children with DS. Similarly, reduced functional connectivity may be evident also in adults between the right SMA complex and basal ganglia (Yang *et al*., 2016) and between the left inferior frontal regions and pre-SMA (Lu *et al*., 2012; 2016). During speech tasks, higher connectivity is reported from the thalamus to the pre-SMA (Lu et al., 2010) and from the inferior parietal and inferior frontal cortex to the SMA complex (Kell *et al*., 2018). Stuttering severity may correlate with SMA activity as well as with its connectivity. Interestingly, speaking under fluency-inducting conditions is associated with the normalization of SMA functioning (Fox *et al.*, 1996). Structural and functional changes of the SMA complex seem to be related to stuttering persistence or recovery (Garnett *et al*., 2018). The age-related decreased in local gyrification of proper SMA and pre-SMA evident in children who do not persist may result in better connectivity between the SMA complex and inferior frontal regions (Garnett *et al.*, 2018). As well, it may represent the consequence of a functional reorganization of the neural system that relies on different and more efficient neural structures thus favoring speech fluency (Busan, 2020).

In the light of the above findings, the SMA complex may not be related to stuttering in a causal way but rather represent a crucial hub that integrates a series of dysfunctional signals from deficient networks involved in speech motor control. Connections with limbic structures (Nachev *et al*., 2008) may further modulate SMA activity. Despite the

present evidence which highlights a pivotal role of the SMA complex in DS, many aspects of its involvement in the pathophysiological mechanism of dysfluent speech are not fully disentangled.

2.4.2 Stuttering as a generalized motor syndrome

As was pointed out in previous paragraphs, stuttering is not related to a single disturbance in a confined cerebral region. Rather, it is the result of widespread neural abnormalities and dysfunctional interactions between different brain networks (Qiao *et al.*, 2017; Daliri and Max, 2015). These encompass brain areas involved in speech processing (Fox *et al*., 1996; Chang *et al*., 2008) and almost all the cortical and subcortical structures that support the selection and execution of motor/speech acts (Sommer *et al*., 2002; Watkins *et al.*, 2008; Lu *et al.*, 2010). Stuttering shares many features with several motor disorders (Sommer *et al.*, 2002; Mulligan *et al*., 2003; Ludlow and Loucks, 2003), and acquired neurogenic stuttering is more frequent after lesions in structures that belong to the motor system rather than after lesions of speech-related areas (Lundgren *et al*., 2010). A full range of neuroimaging, neurophysiological, and behavioral studies suggests that in DS the loss of speech motor control may be only the overt symptom of a more general neuromotor deficit (Ludlow and Loucks, 2003; Preibisch *et al*., 2003; Chang *et al*., 2008; Neef *et al*., 2015b; Busan *et al.*, 2017). Indeed, stuttering is associated with lower white matter integrity also in long range cortical connections to not speech-related muscles (Connally *et al*., 2014) and with impaired manual motor skills (Webster,1990; Smits Bandstra and De Nil, 2007, Daliri *et al.*, 2014).

In this context, non-invasive neurophysiological techniques such as transcranial magnetic stimulation (TMS) have recently provided novel insights into the motor system functioning of people with DS and thus into the pathophysiology of the disturbance (Neef *et al*., 2015a Busan *et al*., 2017). TMS is widely used in the study of the nervous system of numerous motor disorders (Bares *et al*., 2003; Lozeron *et al.*, 2016) providing important insights into the neurophysiology of cortico-spinal and cortico-bulbar pathways as well as into the activity of the intracortical circuits involved in the modulation of motor outputs (Hallett, 2000; Kobayashi and Pascual Leone, 2003). In this light, it is surprising that only few research groups have employed TMS to study the motor functioning of people with persistent developmental stuttering (see for a recent review Busan *et al*., 2017).

Early TMS studies in DS have focused mainly on primary motor cortex representations of not speech-related muscles during no concurrent speech/behavioral tasks (Busan *et al.*, 2017). TMS revealed that adults with DS and fluent speakers usually show opposite patterns of cortical excitability and thus different motor asymmetries (Alm *et al.*, 2013). In people with DS, resting and active motor thresholds of the left primary motor cortex representations of hand muscles are abnormally higher than those of fluent speakers (Sommer *et al*., 2003) but also when compared to their own right (Alm *et al*., 2013). Enhanced motor thresholds may reflect dysfunctions in the cortico-spinal connections (Sommer *et al.*, 2003). This is also supported by lower stimulus-response curves that may be related to the reduced number of cortical projecting neurons and/or to the lower strength and recruitment of the left cortico-spinal tract (Busan *et al*., 2013; see also Connally *et al*., 2014). Interestingly, no difference is evident when considering the interplay between left and right motor regions as suggested from the normal interhemispheric inhibition and ipsilateral cortical silent period duration (Sommer *et al*., 2009). In the hand motor cortex, the mechanisms of intracortical inhibition (short-interval intracortical inhibition and cortical silent period duration) and facilitation are not evidently altered (Sommer *et al*., 2003; Busan *et al.*, 2013). However, some indexes of intracortical motor functioning (e.g. cortical silent period) often correlates with stuttering severity (Busan *et al*., 2013; Busan et *al.*, 2016) thus supporting the suggestion that dysfluencies could be only the most evident symptom of a more generalized motor deficit (Busan *et al.*, 2017). In this regard, stuttering severity negatively correlates with the duration of the cortical silent period obtained when stimulating the right hemisphere (Busan *et al*., 2013) and positively correlates with that obtained when stimulating the left one (Busan *et al.*, 2016). The relation between cortical silent period duration and stuttering is also supported by a combined pharmacological/TMS study that revealed improvement in stuttering-associated movements and spasms in concomitance to the reduction of the left-hemisphere cortical silent period duration after the administration of paroxetine (Busan *et al*., 2009). Abnormal modulation of primary motor cortex excitability of not speech-related muscles may be evident also during speech/behavioral tasks. For example, left primary motor cortex of hand muscles is excessively facilitated during spontaneous speech and abnormally reduced during non-verbal orofacial movements in DS (Sommer *et al.*, 2019). The application of repetitive TMS (rTMS) suggests that different premotor influences on motor outputs may be present between people with DS and fluent speakers. The synchronization of auditory-paced finger movements normally relies on the activity of left

dorsolateral premotor cortex (PMd) as evidenced from synchronization disruptions after rTMS over this area (Neef *et al*., 2011b). This is not true for people with DS in which, conversely, this synchronization is disrupted only after the administration of rTMS over the right PMd (Neef *et al*., 2011b). In this case, the involvement of the right PMd in this process probably reflects a compensatory mechanism for the deficient left hemisphere connections and subcortical-cortical interactions and is consistent with the abnormal functional overactivations that are often reported in adults with DS (Fox *et al.*, 1996; Braun *et al*., 1997).

Distinct brain asymmetries are evident also when considering the transcranial magnetic stimulation of primary motor cortex representations of speech-related muscles such as the tongue (Barwood *et al*., 2013; Busan *et al*., 2016). Similarly to what was observed in hand primary motor cortex representations, cortico-bulbar excitability of tongue muscles is usually higher in the left hemisphere of fluent speakers while in people with DS motor excitability is increased in the right hemisphere and decreased in the left one (Barwood *et al*., 2013; Busan *et al*., 2016). Different motor asymmetries are evident also when considering indexes of inhibitory function such as cortical silent period threshold, which is abnormally higher in the left hemisphere of people with DS (Busan *et al*., 2016). Paired-pulse and single-pulse protocols revealed the existence of an altered balance between excitatory and inhibitory circuits underling primary motor cortex representation of tongue muscles (Neef *et al*., 2011a; Busan *et al*., 2016). Intracortical facilitation is bilaterally reduced in people with DS while short interval intracortical inhibition is delayed, especially in the right hemisphere (Neef *et al*., 2011a). Prolonged contralateral silent period durations recorded when stimulating the left hemisphere also suggest the existence of an abnormal higher level of intracortical inhibition in these circuits (Busan *et al.*, 2016). Dysfunctional activity of inhibiting interneurons at rest, in DS, may be related to white matter deficiencies (Sommer *et al*., 2002; Connally *et al*., 2014) and to the functional abnormalities that are often reported in DS (Etchell *et al*., 2018). More properly it may be the result of the aberrant interactions between cortical and subcortical structures in modulating the final level of excitability of the motor output (Neef *et al*., 2011a; Busan *et al*., 2016). In this context, the application of TMS on tongue motor cortex during concomitant speech tasks suggests the existence of different speech dynamics and abnormal speech/motor preparation in DS. Conversely to what was observed in matched fluently speaking controls, speech production of people who stutter is associated with reduced tongue motor cortex excitability prior to speech onset (Whillier *et al*., 2018) and

during the transition between speech gestures especially in the left hemisphere (Neef *et al*., 2015b).

Collectively, this evidence demonstrates that the involvement of the motor system in developmental stuttering is more wide and critical than previously thought and strongly supports the theory that dysfluencies may be only the most evident symptom of a more general motor dysfunction (Busan *et al*., 2017). However, TMS studies have investigated basic aspects of the motor system mainly at rest or during very simple speech/motor tasks, and many important aspects of the DS motor functioning are still obscure.

3 Aim of the thesis

This dissertation aims to further explore and describe the neurophysiological substrate that underlies the brain functioning of adults with persistent developmental stuttering in order to make contributions that could provide novel insights into this incompletely understood neurological motor disturbance.

A series of neural markers suggests that stuttering may be the result of deficient neural dynamics between brain networks that support motor behavior, speech processing, and cognition. Dysfunctional activity within brain structures associated with motor planning, execution, and control is evident also in the absence of speech tasks thus suggesting that dysfluencies may be only the overt symptom of a more subtle motor disorder. In this context, despite the crucial role of the motor system in DS only a basic knowledge of its neural correlates is available. As a consequence, the employment of non-invasive neurophysiologic and brain stimulation techniques can provide useful information about more complex mechanisms such as those involved in action implementation during motor/speech preparation and control and to obtain a broader understanding of DS brain dynamics such as the neural exchange between different brain networks during motor tasks

By using multimodal non-invasive investigation tools (e.g. neurophysiological recordings and brain stimulation techniques), this thesis intends to fill a series of research gaps that specifically include:

- the characterization of temporal neural dynamics related to the activation of a "central hub" in DS such as the supplementary motor complex by means of transcranial magnetic stimulation and electroencephalography (TMS/EEG co-registration; *Study 1*). TMS will allow to "activate" the SMA in a controlled manner, while the EEG co-registration will allow to individuate abnormal patterns of neural connectivity in persistent stuttering;
- the investigation of neural dynamics characterizing the interplay between different muscular districts when involved in motor tasks (*Study 2*). TMS will allow to characterize the cortico-spinal excitability and intracortical functioning of primary motor cortex networks, when involved in motor implementation and execution;
- the characterization of the time-course neural changes in the preparation period that precedes the execution of a volitional motor act, and the better understanding

of the influence of “arousal” (i.e. the presence of an “audience”) on brain dynamics associated with speech preparation and production, in DS (Study 3). Magnetoencephalography (MEG) will allow to identify brain networks that may have a “negative” modulatory effect on speech fluency, investigating if abnormal sensorimotor processing of people who stutter is further influenced by external factors, such as social “pressure”.

These studies have sought to unravel novel neural markers of DS that could be helpful to define more focused and effective rehabilitation treatments for affected individuals.

Study 1 and *Study* 2 already allowed to publish parts of findings in international “peer-reviewed” scientific journals in the field (*Busan P., Del Ben G., Russo L.R., Bernardini S., Natarelli G., Arcara G., Manganotti P., Battaglini P.P., 2019. Stuttering as a matter of delay in neural activation: a combined TMS/EEG study. Clinical Neurophysiology, 130(1):61-76. doi: 10.1016/j.clinph.2018.10.005; Busan P., Del Ben G., Tantone A., Halaj L., Bernardini S., Natarelli G., Manganotti P., Battaglini P.P. 2020. Effect of muscular activation on surrounding motor networks in developmental stuttering: A TMS study. Brain and Language, 205, 104774. doi: 10.1016/j.bandl.2020.104774*).

On the other hand, at the moment of the writing of this thesis, the recruitment and data acquisition of *Study 3* have been completed, while data analysis still needs to be determined. Consequently, preliminary data will be presented in this work.

4 Study 1

4.1 Introduction

Abnormal activations of cortical and subcortical structures (Fox *et al*., 1996; Watkins *et al*., 2008; Ingham *et al*., 2012) and widespread white matter deficiencies (Chang *et al*., 2008; Connally *et al.*, 2014) are probably the strongest neural signatures of developmental stuttering and may be causally related to speech dysfluencies. However, classical neuroimaging approaches failed to shed light on the precise temporal interactions of brain dynamics behind the reduced activity of left-hemisphere inferior frontal regions and homologues right-hemisphere overactivations. Their reciprocal relationships with white matter deficiencies and abnormal basal ganglia functioning are also still obscure. A brain region that may act as a strong connection "hub", in DS, receiving and elaborating information from the just mentioned neural networks (likely in a bidirectional way), is the supplementary motor area (SMA; see Busan, 2020). The dysfunctional activity of the supplementary motor area in stuttering has been frequently reported in the literature (Brown *et al*., 2005; Budde *et al*., 2014) however, only recently it has been proposed as a new neural marker of DS (Neef *et al*., 2015a; Busan, 2020). The supplementary motor area is crucial in the generation of internally driven motor acts (Narayana *et al*., 2012) and in the preparation of complex motor sequences such as speech (Nachev *et al*., 2008). In addition, it is functionally connected with cerebral structures involved in planning and execution of motor acts (Ruan *et al*., 2018), with inferior frontal regions (Catani *et al*., 2012), and with areas involved in decision making and planning of behaviors (Zhang *et al*., 2012). In this light, SMA is critical in fluent speech production and may represent a central node in the pathophysiological mechanisms that underlie DS since it may be requested to integrate neural signals from a series of dysfunctional networks (Yang *et al*., 2016; Chang *et al*., 2018; Busan, 2020) before motor program release.

Based on these pieces of evidence, the present study aims to take advantage of the combination of transcranial magnetic stimulation and electroencephalography (TMS/EEG) to shed the first light on the "whole brain" neural temporal dynamics related to the activation of the supplementary motor complex in adults with persistent DS. More specifically, considering the previously available evidence (see Chapter 2), we hypothesize that the TMS-induced activation of the SMA of people who stutter will allow to individuate defective patterns of brain connectivity (with particular attention to

abnormal temporal dynamics, individuated by means of EEG), thus helping to elaborate new suggestions for stuttering treatment.

TMS/EEG is a very useful approach to fulfill this objective since it allows to study the propagation of neural signals from the perturbated structures to almost the entire cerebral cortex (Ilmoniemi *et al*., 1997) also providing very important information about the reactivity of the stimulated regions as well as on their functional and effective connectivity (Mininussi *et al*., 2013).

4.2 Materials and methods

4.2.1 Participants

Twenty-eight right-handed male adults were recruited for this study. Thirteen (age range 24–47 years, mean 32.9 years, standard deviation [SD] ± 8.3) were stutterers with a history of DS since childhood while the others fifteen (age range 22–48 years, mean 30.4 years, SD ± 7.2) were fluent speakers (FS) with no self-reported history of stuttering or other speech disorders. All participants were Italian native speakers and none of them reported a history of major neurological disorders, psychiatric disorders or severe brain injuries. In addition, none of them showed neurological abnormalities (other than stuttering in the DS group), was under pharmacological treatment with psychiatric medications or used to assume psychoactive drugs at the time of the study. Groups (DS; FS) were matched for variables such as age, handedness, smoking habits, level of education, amount of musical training and physical activity, migraine diagnosis, presence of depressive symptoms. Participants were screened for risks related to TMS (Rossi *et al*., 2009; Rossi *et al*., 2021). Participants gave a written informed consent and authorized the use and process of personal data in compliance with the Italian Law. The experimental procedure was approved by the local Ethics Committee and was in accordance with the "World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects" and recent TMS guidelines (Rossi *et al*., 2009; Rossi *et al.*, 2021). Participants were able to leave the experiment in any moment without giving reasons to researchers and did not receive any compensation for participating in this study.

Table 1 summarizes demographic characteristics of participants, in both groups.

Characteristics/Groups	DS	FS	p-value
Age	32.9 ± 8.3	30.4 ± 7.2	p = 0.39
Education	17.3 ± 3.6	15.7 ± 2.2	p = 0.34
Handedness	84.4 ± 12.2	85.4 ± 12.6	p = 0.84
Smoke habits	0.24 ± 0.43	0.2 ± 0.41	p = 0.67
Migraine	0.1 ±0.28	0.07 ± 0.26	p = 0.58
Musical training	0.26 ± 0.42	0.23 ± 0.41	p = 0.60
Physical training	6/7	12/3	p = 0.14

Table 1. **Main characteristics of participants**. Data are represented reporting mean ± standard deviation. Levels of smoking habits, migraine and musical training were standardized on a 0 to 1 scale. Physical training is reported indicating the amount of "active" and "inactive" participants.

4.2.2 Behavioral/cognitive assessment

Handedness was assessed through the Edinburgh Handedness Inventory (Oldfield, 1971). Cognitive and behavioral evaluation was assessed by the administration of the Italian adaptation of the Beck Depression Inventory-II (BDI-II; Beck *et al*., 1996). Speech attitudes were evaluated by administering the Italian adaptation of the adult form of the Communication Attitude Test (BigCAT; Vanryckeghem and Brutten, 2012). Stuttering severity was assessed through the Stuttering Severity Instrument-4 (SSI-4; Riley *et al*., 2009) amongst DS group members only. DS participants were audio and video recorded during about 3-5 minutes of spontaneous speech and a reading task of the same written text. Stuttering severity was assessed in terms of frequency, duration, physical concomitants, and naturalness of the individual's speech. Fluently speaking controls were interviewed by a trained researcher to exclude the presence of undetected stuttering or other speech disorders.

4.2.3 Experimental setup

4.2.3.1 Transcranial magnetic stimulation

Participants were seated on a comfortable chair in a relaxed position and were asked to keep their eyes open. Self-adhesive disposable electrodes (Ag/AgCl) were bilaterally placed on a tendon belly montage over the *first dorsal intraosseous* (FDI) muscle and a ground electrode was placed on the right forearm. Participants wore a lycra cap on which a grid of 10 mm spaced point was drawn to better individuate the position on the scalp

that allowed to obtain the most evident and reliable motor evoked potentials (MEPs). TMS (Medtronic MagPro R30) biphasic stimuli were administered by applying a "figure-of-eight" shaped stimulation coil (Medtronic C-B60 – wing outer diameter about 75mm; antero-posterior direction of the first phase of the current in the coil) on the participants' scalp at the primary motor cortex level. During the stimulations, the coil was manually positioned and maintained on the scalp with the handle pointing backwards at a 45° angle with respect to the medial longitudinal fissure. Electromyographic (EMG) activity was recorded using a digital band-pass filtering of 20-2000 Hz (sampling rate 8000 Hz). The coil was positioned on the fronto-central region of the subject's scalp and stimuli were applied in order to identify the motor representation of the contralateral FDI muscle on the basis of MEP onset on the EMG trace. Resting motor threshold (RMT) was individuated as the minimum stimulation intensity resulting in a MEP of at least 50 μV in half of 8-10 consecutive trials, when stimulating the left hemisphere FDI motor representation (also the RMT of the right hemisphere FDI motor representation was recorded to verify that significant differences were not evident, between groups and hemispheres; always $p > 0.1$). The cortical target corresponding to the SMA complex was individually identified by using a system based on scalp measures (nasion-inion/bi-auricular distances) and EEG coordinates and then marked on the cap. Self-adhesive disposable electrodes (Ag/AgCl) were bilaterally placed also on several other muscles, including the *abductor digiti minimi*, *abductor pollicis brevis*, *trapezius*, *tibialis anteriori*, and on muscular districts of the forearm, the biceps, and deltoids. TMS single pulses were administered over this point at the 100% of the left-hemisphere resting motor threshold of the left FDI to assure that it was not possible to evoke motor potentials from the recorded muscles bilaterally.

4.2.3.2 Neuronavigation

The correct stimulation spot was individuated on each participant using a computer-assisted neuronavigation system (Visor-2, ANT NEURO B.V., The Netherlands) to accurately target TMS stimuli during the TMS/EEG co-registration. Participants were seated on a comfortable chair with a tissue EEG cap (Electro-Cap B.V., The Netherlands) on their scalp that would be later used for the EEG acquisition. A standard magnetic resonance of an healthy adult was used to create the head model. Markers for nasion and left and right periauricular points were recorded and about 200 sample points were

digitized on the participant's head to refine the registration and obtain a realistic head model. Based on the coordinates inferred by Zhang and colleagues (Zhang *et al*., 2012), MRI neuronavigation was targeted to the SMA "complex" to allow effective stimulation of the bilateral "proper" SMA and pre-SMA (MNI coordinates: x = 0, y = 6, z = 66; Talairach coordinates: x = 0, y = 9, z = 60). The individuated point on the scalp was marked on the EEG cap in order to easily and accurately identify it during the stimulation sessions. The putative maximal current (V/m) delivered to the cortex by the coil when placed on the stimulation point was calculated through the software of the neuronavigation system.

4.2.3.3 EEG recordings

The electroencephalographic activity was recorded by 31 electrodes equally distributed on the cap as follows: Fp1, Fp2, Fpz, Afz, F7, F8, F3, F4, Fz, FC1, FC2, FC5, FC6, Cz, C3, C4, T3, T4, CP1, CP2, CP5, CP6, P3, P4, P7, P8, Pz, POz, O1, O2, Oz. The ground electrode was placed below OZ while the reference electrode was placed on the nose with a piece of surgical tape. Two additional self-adhesive Ag/AgCl electrodes were placed on the outer canthus and on the infraorbital ridge of the right eye to record eye blinks and other ocular movements. Electrode impedances were reduced using electroconductive gel and kept below 5-10 kΩ. Electrode wires were arranged to reduce the effect of the TMS magnetic field on the recordings (Sekiguchi *et al*., 2011). The EEG signal was acquired using a BASIS BE (EBNeuro, Italy) amplifier and digitally stored using the MIZAR-SIRIUS system (Galileo NT software; EBNeuro, Italy). The EEG was acquired in a direct current (DC) mode and the sampling rate was set at 4096 Hz to further reduce the magnitude of the TMS artifact. The operational range of the amplifier was set at ± 65.5 mV to limit its saturation.

4.2.3.4 TMS/EEG co-registration

Participants were seated on a comfortable chair for the entire duration of the procedure and wore earplugs to reduce the acoustic cerebral activation induced by the TMS. During the stimulations participants were asked to place their chin on a customized chinrest, to keep their eyes closed (to avoid systematic TMS-induced ocular artifacts), and to avoid any systematic cognitive activity (such as counting). The C-B60 coil was placed on the participants' scalp over the individual SMA spot, perpendicularly to the interhemispheric

fissure with the handle pointing backwards. The correct position of the coil was systematically checked during the stimulation and maintained by the experimenter. A piece of foam of about 5mm of thickness was placed between the coil and the EEG cap to reduce the somatosensory activations induced by the stimulation (Massimini *et al*., 2005). Single pulse TMS was delivered at the 100% of the individual resting motor threshold of the left primary motor cortex representation of the FDI muscle. Participants underwent 3 blocks of real TMS alternated by 3 blocks of sham TMS. Each block consisted in about 50-60 single pulses with an interstimulus interval of about 2-8 seconds. Sham stimulation was realized by adding a piece of wood of about 30mm of thickness between the C-B60 coil and the EEG cap (foam was always in contact with the scalp). This procedure was done in order to avoid the magnetic field to reach the cerebral cortex and at the same time maintain the identical sound click produced by the TMS. Sham stimulation was administered in order to obtain a model of the brain activity evoked only by the somatosensory activations and sound click produced by the real TMS. Participants were not aware of the type of stimulation delivered during the blocks. The maximum electric field induced in the cortex was significantly different when comparing real and sham stimulation in both groups ($p < 0.001$). No significant difference was evident between groups when considering the maximum electric field induced by real TMS, sham, as well as the "net" effect of the magnetic stimulation on the cortex ($p > 0.1$).

4.2.3.5 EEG pre-processing

EEG recordings were processed offline. The procedures were carried out with the commercial software Neuroscan (Compumedics Neuroscan Inc., El Paso, USA) and with free software EEGLAB (Delorme and Makeig, 2004) and erpR (Arcara and Petrova, 2017). Data were digitally filtered applying a low pass IIR filter (edge at 200 Hz) EEG traces were visually inspected, real and sham TMS stimuli were marked on EEG traces and the continuous files were segmented in epochs of 700 ms locked on the TMS delivery (-200ms;+500ms). Epochs were visually inspected and those with excessively noisy EEG o evident artifacts (ocular or muscular artifacts, EEG drifts, etc.) were discarded and were not considered for subsequent analysis. The remaining artifacts were removed performing the independent component analysis (Jung *et al*., 2000). Bad electrodes were then detected and successively interpolated. Data were divided by conditions and re-referenced (for each participant) to a common average reference. After epoch averaging, a "linear de-trend"

function was applied to further reduce the residual TMS artifacts, realigning the traces to the baseline. Grand average transcranial evoked potentials (TEPs) subdivided per groups (DS vs FS) and conditions (real TMS vs sham TMS) were visually inspected using a butterfly plot representation. 5 time-windows of interest after the TMS pulse were identified for further analysis as follows: 36-65 ms, 65-144 ms, 144-256 ms, 256-350 ms, 350-500ms. The first 35 ms after the stimulation resulted with a residual TMS artifact, thus lowering the reliability of recorded potentials.

4.2.3.6 Source reconstruction

Spatio-temporal source reconstruction of the TEPs components obtained from both groups after real and sham TMS was computed using standardized low resolution electromagnetic tomography (sLORETA http://www.uzh.ch/keyinst/loreta.htm, Pascual-Marqui, 2002). The head model consisted in a brain volume partitioned in 6239 voxels at 5 mm spatial resolution (Fuchs *et al*., 2002; Mazziotta *et al*., 2001) restricted to cortical gray matter, as determined by the probabilistic Talairach's atlas (Lancaster *et al*., 2000). The position of EEG electrodes was superimposed on the head model using the MNI152 scalp (Jurcak *et al*., 2007; Öostenveld and Präamstra, 2001). Anatomical labels such as lobes, gyri and Brodmann Areas (BAs) were reported in MNI space. A regularization factor calculating the average TEPs signal-to-noise ratio of each temporal window was applied in order to reduce localization errors. Source reconstruction was performed also in the time window from -200 ms to -10 ms prior to TMS delivery in order to control for possible unspecific effects.

4.2.3.7 Statistical analysis

Behavioral data were compared using Student's *t*-test (normally distributed and homogenous data), Welch's *t*-test (normally distributed but not homogeneous data), Mann-Whitney non-parametric test (not normally distributed data), or Chi-square statistic (with Yates correction, categorical data). Hierarchical levels of analysis were performed on TEPs. A descriptive analysis of amplitudes and latencies was performed from electrodes placed around the stimulation spot (Cz; Fz; FC1; FC2). Then, voxel-by-voxel comparison of EEG sources (real TMS vs Sham TMS) was assessed for each group with non-parametric statistical mapping (SnPM; Nichols and Holmes, 2002) implemented in the LORETA-Key software. Statistical analysis was computed using t-statistics and log of

F-ratio on mean neural activity of identified time-windows of interest, to obtain comprehensive patterns of the activations elicited by the TMS. Regions of interest (ROIs – 15 mm radius) were bilaterally defined for each condition (real vs. sham TMS) in both groups (DS vs FS) by individuating their center of maximal activation in MNI coordinates. Sham TMS activity was subtracted from real TMS activity in each ROI and the results were compared between groups using Student's *t*-test and Welch's *t*-test. Time frame by time frame analysis and mean neural signal analysis were performed. Significant activations of at least 9 consecutive time frames (i.e. > 2 ms, thus resulting in a biologically plausible activation, not less than the duration of an action potential; see Lodish *et al.*, 2000) were further considered and clustered to implement permutation/randomization tests (9999 randomizations -see Premoli *et al.*, 2014; Zanon *et al.*, 2018-; an FDR procedure was applied to the findings, in order to face with multiple activations). The findings were further characterized by providing effect sizes (absolute values), using *Hedges' g*/*Cohen's* $d_{unbiased}$ (Hedges and Olkin, 1985, Cohen, 1988, Ellis, 2010; $0.2 < d_{unbiased} < 0.5$ = small effect; $0.5 < d_{unbiased} < 0.8$ = medium effect; $d_{unbiased} > 0.8$ = large effect). Sources of the baseline activity (-200 ms to -10 ms prior to TMS delivery) were also compared as a control analysis, between groups and conditions, by applying similar procedures. A $p < 0.05$ was considered significant. Finally, a correlation analysis was performed to evaluate if relations among Stuttering Severity Instrument-4 (SSI-4) indexes and differences between groups in source analysis (ROIs) were present (considering mean neural activity and time frame by time frame analysis; in this last case, data were considered only if at least nine consecutive time frames were significantly related, i.e. > 2 ms). Pearson's correlation (r) was used ($p < 0.05$, uncorrected).

4.3 Results

4.3.1 Behavioral/cognitive assessment

Stuttering severity was classified as very mild in two DS participants, mild in three, moderate in five, severe in three.

SSI-4 scores and the corresponding BigCAT score are reported in Table 2.

DS Participant	SSI-4 score	Percentile	Classification	BigCAT
A	12	1-4	Very mild	7
B	13	5-11	Very mild	7
C	18	12-23	Mild	15
D	21	24-40	Mild	9
E	23	24-40	Mild	25
F	25	41-60	Moderate	32
G	28	61-77	Moderate	14
H	30	61-77	Moderate	32
I	31	61-77	Moderate	32
J	31	61-77	Moderate	33
K	32	78-88	Severe	32
L	32	78-88	Severe	32
M	36	89-95	Severe	27

Table 2. Scores obtained from the Stuttering Severity Instrument-4 (SSI-4) and the BigCAT in DS group.

BigCAT showed a statistically significant difference between DS and FS participants thus revealing a negative attitude toward speech and speech abilities in the DS group ($p<0.001$). Data from BigCAT and BDI-II are reported in Table 3.

Characteristics/Groups	DS	FS	p-value
BigCAT	22.9 ± 10.7	3.9 ± 3.3	**p < 0.001**
BDI-II	4.9 ± 5.3	2.9 ± 4.0	p > 0.1

Table 3. Cognitive profile of participants. Data are represented reporting mean ± standard deviation. Significant differences are reported in bold.

4.3.2 TMS - Evoked Potentials

Real TMS resulted in an average of 93.2 (SD ± 17.8) accepted epochs in DS group and 84.7 (SD ± 15.2) epochs in the FS group. Sham TMS resulted in an average of 95.7 (SD ± 14.6) accepted epochs in the DS group and 89.1 epochs (SD ± 11.8) in the FS group.

There were no significant differences between conditions nor between groups in the number of accepted epochs ($p > 0.1$). Transcranial magnetic stimulation evoked potentials were obtained after real and sham TMS in both groups. A series of positive and negative deflections was recorded starting from few milliseconds after the stimulation in recording electrodes (Fig. 1). The strongest responses were recorded in electrodes placed around the point of stimulation (Cz; Fz; FC1; FC2) in which five components were evident. More specifically, two positive components (downward deflections) were observed at approximately 60 ms and 180 ms (P60 and P180 respectively) after TMS delivery. Three negative components (upward deflections) were evident at approximately 45 ms, 100 ms, and 280 ms (N45, N100, and N280 respectively) post stimulus. Thus, five time windows of interest were identified after the administration of the TMS in the average evoked activity of each electrode as follows: 36-65 ms, 65-144 ms, 144-256 ms, 256-350 ms and 350-500 ms.

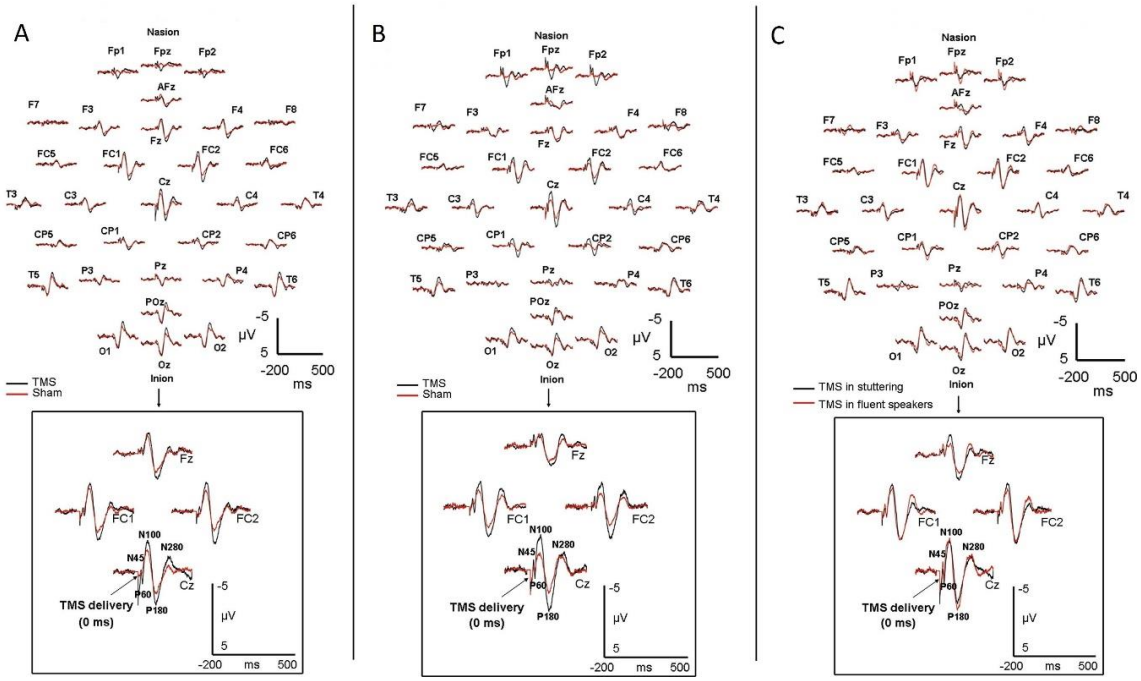


Fig. 1. **Distribution of the TMS-induced EEG evoked components at the recording sites.** Representation of TMS-evoked potentials by real TMS and sham TMS in the DS group and FS group. (A) Real TMS vs. sham in the DS group; (B) real TMS vs. sham in the FS group; (C) comparison of real TMS in DS vs. FS groups. Electrodes placed around the stimulation hotspot are highlighted to represent the main components of TMS-evoked potentials.

4.3.3 Neural source reconstruction

Spatiotemporal neural source reconstruction in DS and FS highlighted distinctive regions of maximal activations in the defined time windows of interest. Activations obtained by real TMS were always higher than those obtained by sham stimulation in both groups and were generally wider and more distributed in the FS group.

In DS, regions of maximal activations (real TMS vs. sham TMS) were centered in the left inferior frontal gyrus and in the precentral gyrus (BA 6) from 36 to 65 ms post stimulus and in the right precentral gyrus (BA 6) and right prefrontal cortex (BA 10) from 65 to 144 ms. These were followed by maximal activations in regions centered in left frontal lobe (BA 6 and BA 46) from 144 to 256 ms, and in regions close to the left temporal cortex (BA 22) and in the right superior frontal gyrus (BA 6) from 256 to 350 ms. Finally, from 350 to 500 ms maximal activation was evident in the right temporal cortex (BA 38) and in the right frontal cortex (BA 6).

Findings are summarized in Figure 2 and Table 4.

In the FS group, maximal activations (real TMS vs. sham TMS) were evident from 36 ms to 65 ms after the TMS in the right-hemisphere superior frontal gyrus (BA 9 and BA 11) and from 144 ms to 256 ms in regions centered in the right parietal lobe (BA 40) and in the left-hemisphere prefrontal cortex (BA 11). In the subsequent time window, from 144 ms to 256 ms post stimulus, regions of the left superior parietal lobe (BA 7) and those surrounding the right middle temporal cortex (BA 21) were maximally activated. These were followed from 256 ms to 350 ms by maximal activations in the right hemisphere around the postcentral gyrus (BA 43) and in the frontal cortex (BA 6). Successively, from 350 ms to 500 ms, maximal activation was evident in the middle temporal cortex (BA 21) and the supramarginal gyrus (BA 40) of the left and right hemisphere respectively.

Findings are summarized in Figure 3 and Table 5.

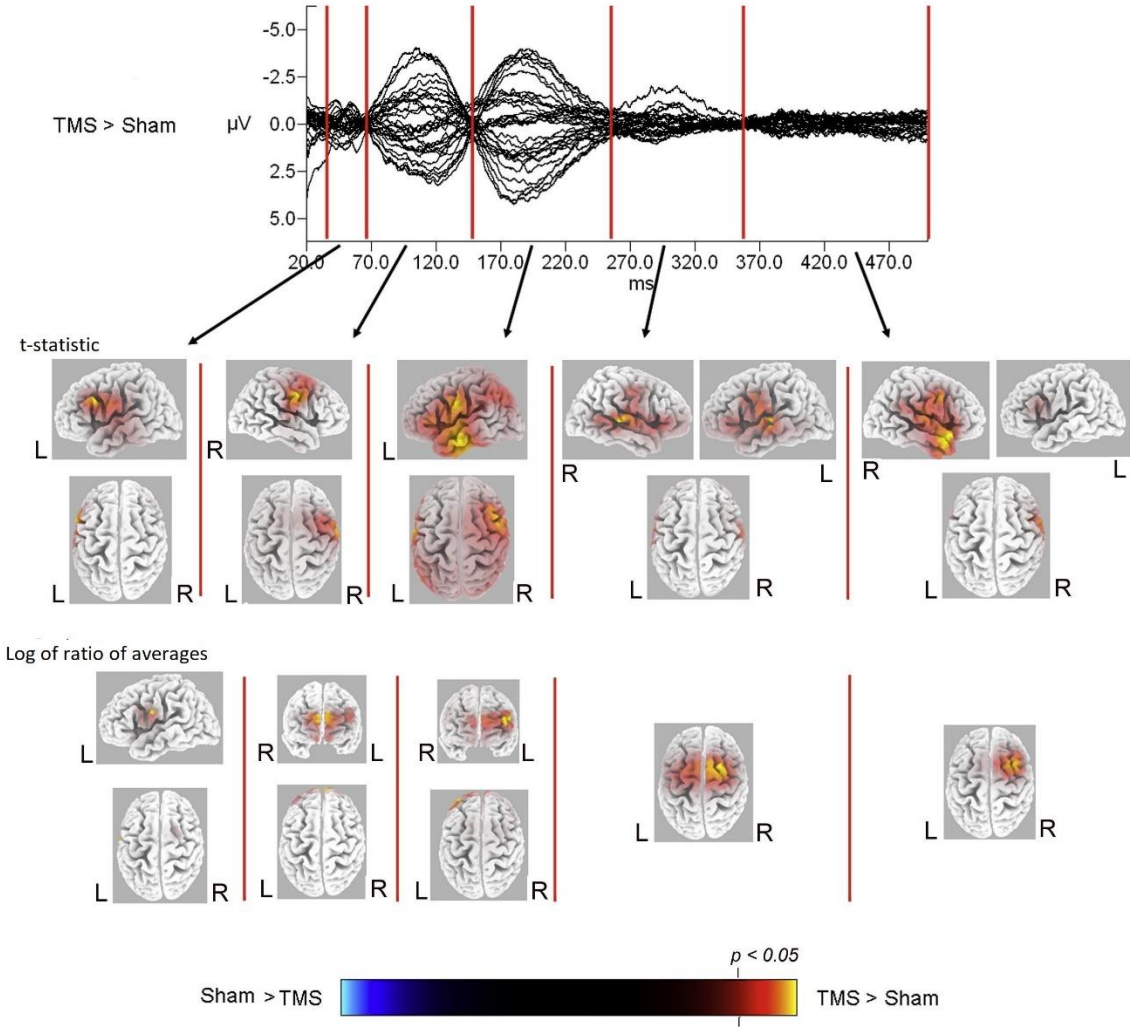


Fig. 2. Current source distribution in DS. Representation of significant neural sources highlighted in the DS group when comparing real TMS to sham TMS in the time windows of interest (mean neural activity).Significant sources obtained by t-statistic and log of F-ratio are reported. L = left hemisphere, R = right hemisphere.

DS - Mean neural activity (sLORETA)						
Time window of interest	***Maximal activation (BA; MNI x, y, z coordinates)***		***Other brain regions activated (BA; left/right)***		***Total number of voxels***	
	t-statistic	***Log of ratio of averages***	***t-statistic***	***Log of ratio of averages***	***t-statistic***	***log of ratio of averages***
36-65 ms	Left inferior frontal gyrus (6L; -60, 10, 30)	Left precentral gyrus (6L; -65, -5, 25)	1L, 2L, 3L, 4L, 8L, 9L, 13L, 20L, 21L, 22L, 36L, 37L, 38L, 40L, 41L, 42L, 43L, 44L, 45L, 46L, 47L	3L, 4L, 9L, 22L, 43L, 44L	610 (*max stat.*, p = 0.0014)	21 (*max stat.*, p = 0.026)
65-144 ms	Right precentral gyrus (6R; 60, -5, 35)	Right medial frontal gyrus (10R; 5, 65, 20)	1L/R, 2L/R, 3L/R, 4L/R, 5L/R, 6L, 7L/R, 8L/R, 9L/R, 13R, 20R, 21R, 22R, 23L/R, 24L/R, 25R, 27R, 28R, 31L/R, 32L/R, 33L/R, 34R, 35R, 36R, 38R, 40L/R, 41R, 42R, 43R, 44R, 45R, 46L/R, 47R	9L/R, 10L, 11L/R, 46L	2369 (*max stat.*, p < 0.0002)	163 (*max stat.*, p = 0.0006)
144-256 ms	Left precentral gyrus (6L; -65, -5, 30)	Left middle frontal gyrus (46L;-45, 45, 20)	1L/R, 2L/R, 3L/R, 4L/R, 5L/R, 6R, 7L/R, 8L/R, 9L/R, 10L/R, 11L/R, 13L/R, 17L/R, 18L/R, 19L/R, 20L/R, 21L/R, 22L/R, 23L/R, 24L/R, 25L/R, 27L/R, 28L/R, 29L/R, 30L/R, 31L/R, 32L/R, 33L/R, 34L/R, 35L/R, 36L/R, 37L/R, 38L/R, 39L/R, 40L/R, 41L/R, 42L/R, 43L/R, 44L/R, 45L/R, 46L/R, 47L/R	6L/R, 8R, 9L/R, 10L/R, 11L/R, 19R, 32L/R, 45L, 46R	4920 (*max stat.*, p = 0.0006)	460 (*max stat.*, p = 0.0016)
256-350 ms	Left superior temporal gyrus (22L; -65,-15, 5)	Right superior frontal gyrus (6R; 20, 5, 70)	3L/R, 4L/R, 6L/R, 9L, 21L/R, 22R, 38R, 40L/R, 42L/R, 43L/R, 44L/R, 45L/R, 47L/R	1L, 3L/R, 4L/R, 6L, 8L/R, 9L/R, 24L/R, 31L/R, 32L/R	185 (*max stat.*, p = 0.012)	665 (*max stat.*, p = 0.006)
350-500 ms	Right superior temporal gyrus (38R; 55, 10, -15)	Right middle frontal gyrus (6R; 30, 10, 65)	1R, 2R, 3L/R, 4L/R, 6L/R, 8R, 9L/R, 10R, 11R, 13L/R, 20R, 21L/R, 22L/R, 24R, 25L/R, 27R, 28R, 32R, 33R, 34R, 35R, 36R, 37R, 38L, 39R, 40R, 41R, 42L/R, 43L/R, 44L/R, 45L/R, 46L/R, 47L/R	6L, 8R, 9R, 24R, 32R	1598 (*max stat.*, p = 0.0012)	188 (*max stat.*, p = 0.0028)

Table 4 . Mean neural activations obtained comparing real TMS and sham in DS.

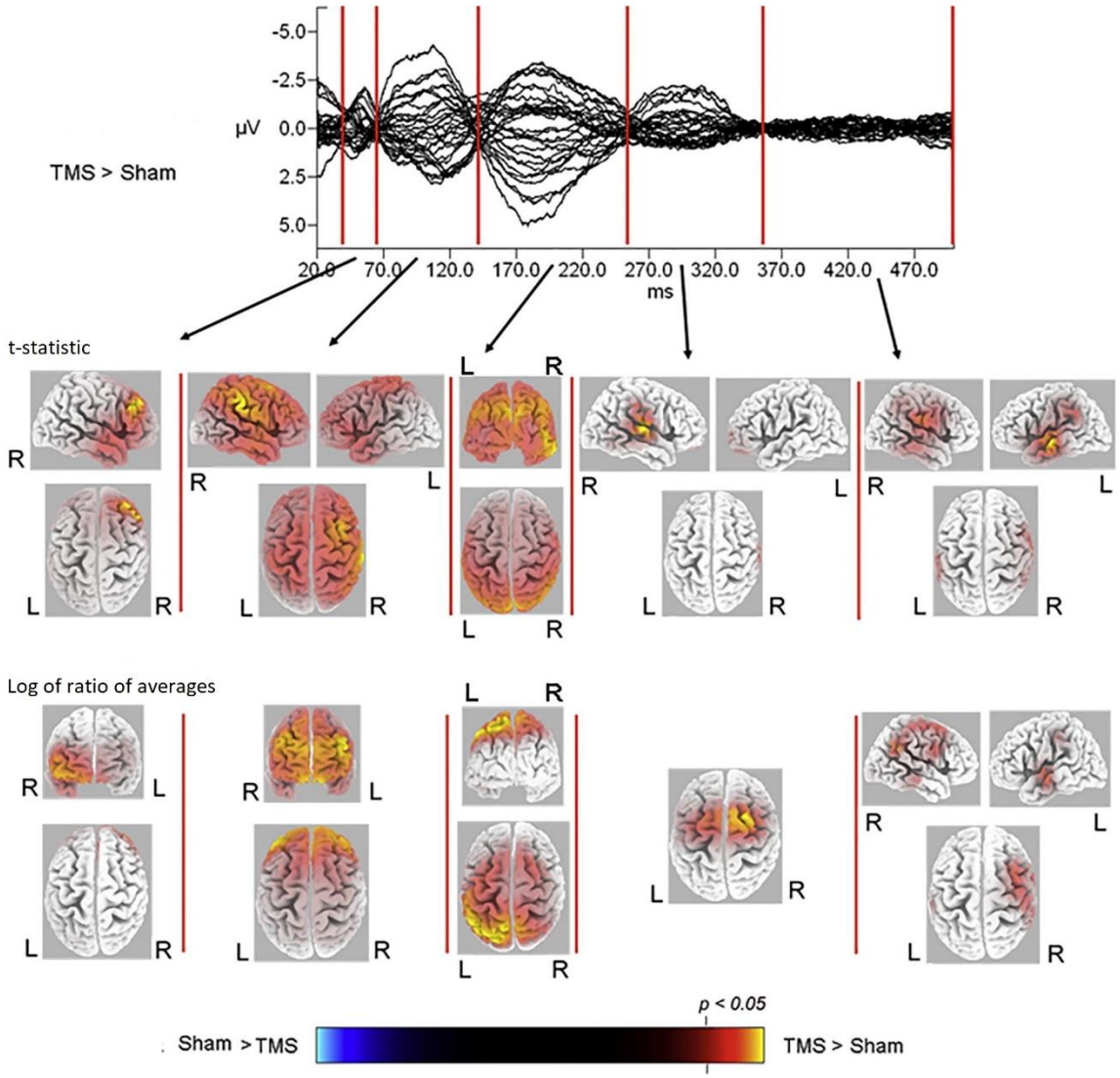


Fig. 3. Current source distribution in FS. Representation of significant neural sources highlighted in the FS group when comparing real TMS to sham TMS in the time windows of interest (mean neural activity).Significant sources obtained by t-statistic and log of F-ratio are reported. L = left hemisphere, R = right hemisphere.

FS - Mean neural activity (sLORETA)						
Time window of interest	***Maximal activation (BA; MNI x, y, z coordinates)***		***Other brain regions activated (BA; left/right)***		***Total number of voxels***	
	t-statistic	***Log of ratio of averages***	***t-statistic***	***Log of ratio of averages***	***t-statistic***	***log of ratio of averages***
36-65 ms	Right superior frontal gyrus (9R; 45, 35, 35)	Right superior frontal gyrus (11R; 30, 55, -15)	4R, 6L/R, 8L/R, 9L, 10L/R, 11L/R, 13R, 20R, 21R, 22L/R, 24L/R, 25L/R, 28R, 32L/R, 33L/R, 34R, 36R, 38R, 42R, 43R, 44L/R, 45R, 46L/R, 47R	9L/R, 10L/R, 11L/R, 13R, 20R, 21R, 22R, 24L/R, 25L/R, 28R, 32L/R, 34R, 36R, 38R, 44R, 45R, 46L/R, 47L/R	1317 (*max stat.*, p < 0.0002)	1024 (*max stat.*, p < 0.0002)
65-144 ms	Right inferior parietal lobule (40R; 65, -30, 40)	Left rectal gyrus (11L; -5, 55, 25)	1L/R, 2L/R, 3L/R, 4L/R, 5L/R, 6L/R, 7L/R, 8L/R, 9L/R, 10L/R, 11L/R, 13L/R, 18R, 19R, 20L/R, 21L/R, 22L/R, 23L/R, 24L/R, 25L/R, 27R, 28L/R, 30R, 31L/R, 32L/R, 33L/R, 34L/R, 35L/R, 36L/R, 37R, 38L/R, 39R, 40L, 41L/R, 42L/R, 43L/R, 44L/R, 45L/R, 46L/R, 47L/R	3L/R, 4L/R, 5L/R, 6L/R, 7L/R, 8L/R, 9L/R, 10L/R, 11R, 13L/R, 20L/R, 21L/R, 22L, 24L/R, 25L/R, 28L/R, 31L/R, 32L/R, 33L/R, 34L/R, 36R, 38L/R, 44L/R, 45L/R, 46L/R, 47L/R	4980 (*max stat.*, p < 0.0002)	2429 (*max stat.*, p < 0.0002)
144-256 ms	Right middle temporal gyrus (21R; 65, -50, -10)	Left superior parietal lobule (7L; -30, -70, 55)	1L/R, 2L/R, 3L/R, 4L/R, 5L/R, 6L/R, 7L/R, 8L/R, 9L/R, 11L/R, 13L/R, 17L/R, 18L/R, 19L/R, 20L/R, 21L, 22L/R, 23L/R, 24L/R, 25L/R, 27L/R, 28L/R, 29L/R, 30L/R, 31L/R, 32L/R, 33L/R, 34L/R, 35L/R, 36L/R, 37L/R, 38L/R, 39L/R, 40L/R, 41L/R, 42L/R, 43L/R, 44L/R, 45L/R, 46L/R, 47L/R	1L/R, 2L/R, 3L/R, 4L/R, 5L/R, 6L/R, 7R, 8L/R, 9L/R, 13L, 18L, 19L/R, 20L, 22L, 23L/R, 24L/R, 27L/R, 28L, 29L/R, 30L/R, 31L/R, 32L/R, 33L/R, 35L, 36L, 37L, 39L/R, 40L/R, 41L, 42L, 43L, 44L, 45L, 46L	5194 (*max stat.*, p = 0.0006)	2503 (*max stat.*, p = 0.0018)

256-350 ms	Right postcentral gyrus (43R; 65, -15, 15)	Right middle frontal gyrus (6R; 30, 10, 65)	1R, 2R, 3R, 4R, 6R, 10L/R, 11L/R, 21R, 22R, 37R, 40R, 42R	NA	194 (*max stat.*, p = 0.01)	1 (*max stat.*, p = 0.023)
350-500 ms	Left middle temporal gyrus (21L; -65, -15, -5)	Right supramarginal gyrus (40R; 65, -50, 30)	1L/R, 2L/R, 3L/R, 4L/R, 5R, 6L/R, 8R, 9L/R, 13L/R, 20L/R, 21R, 22L/R, 24R, 31R, 37R, 38L/R, 39L/R, 40L/R, 41L/R, 42L/R, 43L/R, 44L/R, 45L/R, 46R, 47L/R	1L/R, 2L/R, 3L/R, 4L/R, 6L/R, 8R, 9R, 13R, 20L/R, 21L/R, 22L/R, 24R, 31R, 39R, 40L, 41L/R, 42L/R, 43L/R, 44R, 45R	1637 (*max stat.*, p = 0.0018)	692 (*max stat.*, p = 0.004)

Table 5. Mean neural activations obtained comparing real TMS *vs*. sham in FS.

4.3.4 Regions of interest analysis

Previous analysis allowed to individuate various regions of interest (ROIs) that were used to investigate the existence of significant differences between groups, when the SMA "complex" was stimulated. In addition to the region corresponding to the point of stimulation, 4 ROIs were identified in each of the previously defined time windows, based on the mean neural activity of both groups when comparing real TMS *vs*. sham. ROIs are summarized in Fig. 4 and Table 6.

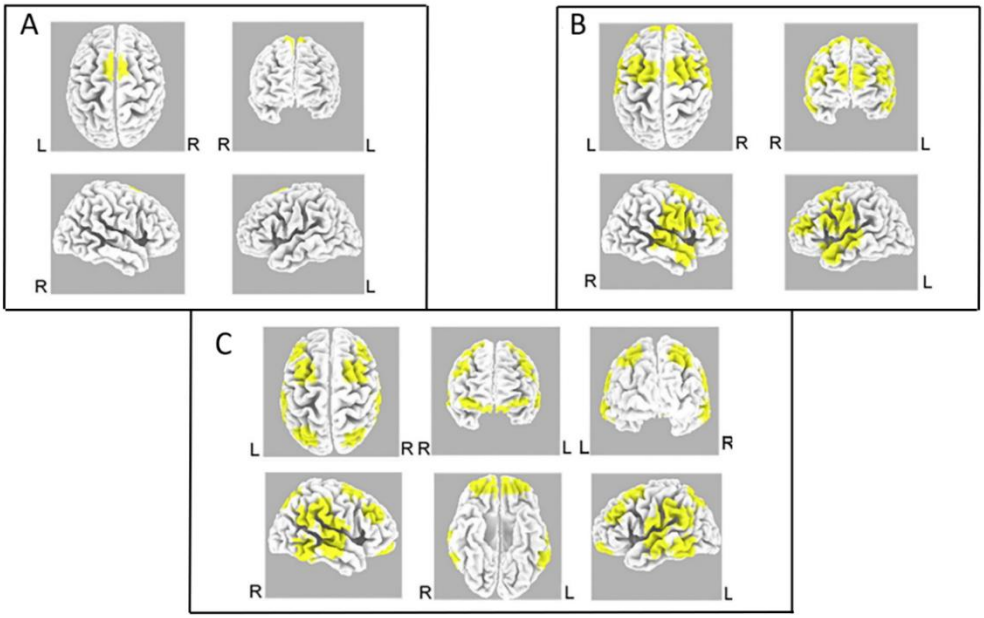


Fig. 4. Regions of interests (ROIs) (A) ROIs corresponding to the stimulation point; (B) ROIs obtained from source analysis in DS; (C) ROIs obtained from source analysis in FS.

Time window of interest (main analysis)	*Center of ROI (BA; MNI x, y, z coordinates)*	*Other BAs in ROI*	*Total voxels of ROI*
36-65 ms (FS, t-statistics, mean activity)	Middle frontal gyrus, BA 9 L (-45, 35, 35) Superior frontal gyrus, BA 9 R (45, 35, 35)	BA 8, 10, 45, 46	31-40
65-144 ms (FS, t-statistics, mean activity)	Inferior parietal lobule, BA 40 L/R (-65, -30, 40; 65, -30, 40)	BA 1, 2, 3	53-58
144-256 ms (FS, t-statistics, mean activity)	Middle temporal gyrus, BA 21 L/R (-65, -50, -10; 65, -50, -10)	BA 19, 20, 22, 37	45
256-350 ms (FS, t-statistics, mean activity)	Postcentral gyrus, BA 43 L/R (-65, -15, 15; 65, -15, 15)	BA 1, 3, 4, 6, 22, 40, 41, 42	52
350-500 ms (FS, t-statistics, mean activity)	Middle temporal gyrus, BA 21 L/R (-65, -15, -5; 65, -15, -5)	BA 22, 41, 42	45-54
36-65 ms (FS, log of ratio of averages, mean activity)	Superior frontal gyrus, BA 10 L/R (-30, 55, -15; 30, 55, -15)	BA 11	39-42
65-144 ms (FS, log of ratio of averages, mean activity)	Rectal gyrus, BA 11 L/R (-5, 55, -25; 5, 55, -25)	NA	24
144-256 ms (FS, log of ratio of averages, mean activity)	Superior parietal lobule, BA 7 L/R (-30, -70, 55; 30, -70, 55)	BA 40	56
256-350 ms (FS, log of ratio of averages, mean activity)	Middle frontal gyrus, BA 6 L/R (-30, 10, 65; 30, 10, 65)	BA 8	37-42
350-500 ms (FS, log of ratio of averages, mean activity)	Supramarginal gyrus, BA 40 L/R (-65, -50, 30; 65, -50, 30)	13, 22, 39	42-48
36-65 ms (DS, t-statistics, mean activity)	Inferior frontal gyrus, BA 9 L/R (-60, 10, 30; 60, 10, 30)	BA 6, 44, 45,46	30-36
65-144 ms (DS, t-statistics, mean activity)	Precentral gyrus, BA 6 L/R (-60, -5, 35; 60, -5, 35)	BA 3, 4	34-39
144-256 ms (DS, t-statistics, mean activity)	Precentral gyrus, BA 6 L/R (-65, -5, 30; 65, -5, 30)	BA 1, 3, 4, 43	4-15
256-350 ms (DS, t-statistics, mean activity)	Superior temporal gyrus, BA 22 L/R (-65, -15, 5; 65, -15, 5)	BA 6, 21, 40, 41, 42, 43	52-55
350-500 ms (DS, t-statistics, mean activity)	Superior temporal gyrus, BA 38 L/R (-55, 10, -15; 55, 10, -15)	BA 13, 21, 22, 47	48-58
36-65 ms (DS, log of ratio of averages, mean activity)	Precentral gyrus, BA 6 L/R (-65, -5, 25; 65, -5, 25)	BA 1, 3, 4, 9, 22, 42, 43, 44	26-46
65-144 ms (DS, log of ratio of averages, mean activity)	Medial frontal gyrus, BA 10 L/R (-5, 65, 20; 5, 65, 20)	BA 9	20-26
144-256 ms (DS, log of ratio of averages, mean activity)	Middle frontal gyrus, BA 46 L/R (-45, 45, 20; 45, 45, 20)	BA 10	30-36
256-350 ms (DS, log of ratio of averages, mean activity)	Superior frontal gyrus, BA 6 L/R (-20, 5, 70; 20, 5, 70)	NA	40-42
350-500 ms (DS, log of ratio of averages, mean activity)	Middle frontal gyrus, BA 6 L/R (-30, 10, 65; 30, 10, 65)	BA 8	37-42
36-500 ms (SMA “complex”, target of stimulation)	Superior frontal gyrus, BA 6 (0, 6, 66)	BA 8	51

Table 6. ROIs individuated when comparing real TMS *vs*. sham in the DS and FS groups.

Significant differences between groups comparing real TMS vs sham TMS were highlighted in almost all of the previously defined time windows of interest.

The FS group resulted in higher activations (with respect to DS) in the ROI corresponding to the point of stimulation (SMA - superior frontal gyrus, BA 6) between 66 ms and 71 ms and from 75 ms to 82 ms after the delivery of the stimulus. The same was evident in the ROI corresponding to the left precentral gyrus (BA 6) between 91 ms and 102 ms, as well as in the left inferior parietal lobule (BA 40) between 99 ms and 101 ms and in the right superior parietal lobule (BA 7) from 149 ms to 152 ms after TMS.

From this moment on, significant differences were mainly evident in the right hemisphere which always resulted in higher activations in the DS group. The right superior temporal cortex (BA 22) was more active from 263 ms to 268 ms and from 273 ms to 280 ms. Enhanced neural activations were evident in the DS group also in the right parietal cortex (BA 43) between 265 ms and 268 ms and from 274 ms to 277 ms after the stimulus. Successively, FS resulted in higher activity of the left middle temporal gyrus (BA21) from 369 to 374 ms after stimulus delivery. Immediately after, the homologous region of the right hemisphere resulted more active in DS between 378 ms and 380 ms. In this context, enhanced neural activity was evident in DS in right superior temporal gyrus (BA 38) between 378 ms and 380 ms and between 425 ms and 427 ms as well as in the right middle frontal gyrus (BA 6) between 454 ms and 462 ms after the stimulus. Finally, greater activity was evident in DS in the ROI corresponding to the right superior frontal gyrus (BA 6) from 456 ms to 463 ms in a brain region that overlaps the point of stimulation.

All statistics and results are summarized in Figure 5 and Table 7.

4.3.5 Control analysis

Results obtained from the ROI analysis were used to verify if similar findings were evident when considering baseline activity (from -200 ms to -10 ms before TMS delivery).

No significant differences were present between groups.

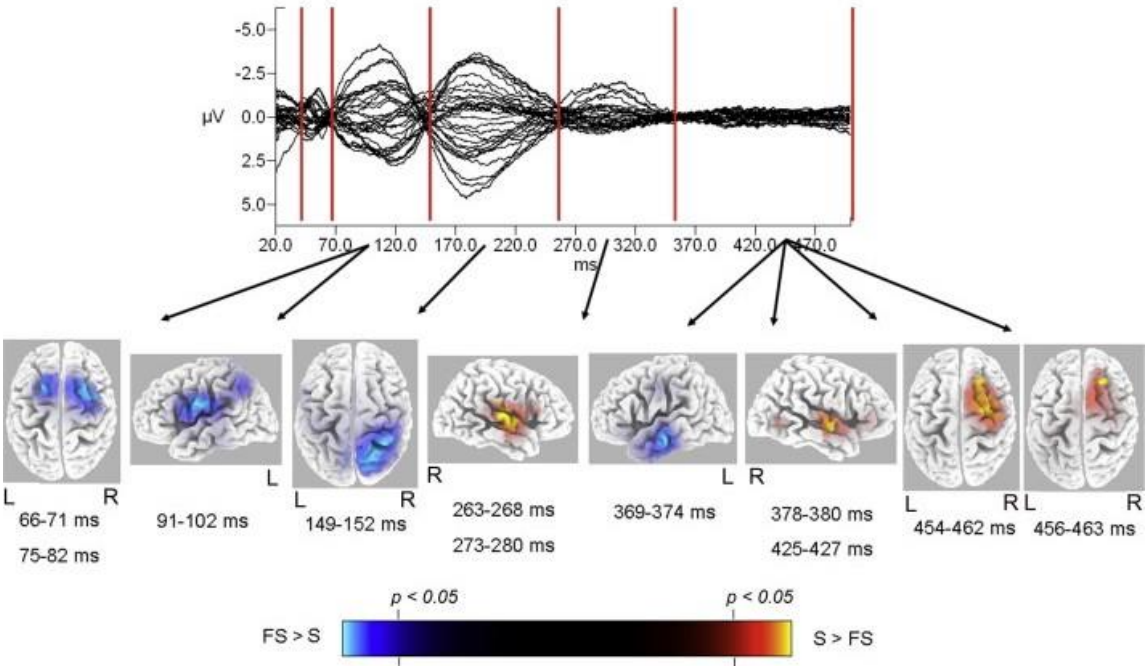


Fig. 5. ROI - Significant differences between DS and FS in the time windows of interest. Activations are reported by using relative scales to represent voxels that were significantly activated ($p < 0.05$, corrected); L = left hemisphere, R = right hemisphere.

Time window of interest	***Center of ROI (BA; MNI x, y, z)***	*Mean neural activity (TMS – sham)*	***Statistics***	***Effect***
66-71 ms **75-82 ms**	Superior frontal gyrus (6; 0, 6, 66)	0.097 ± 0.21 (DS) 0.441 ± 0.5 (FS); 0.166 ± 0.32 (DS) 0.668 ± 0.68 (FS)	permutation test, p = 0.023; t(26) = 2.39, p = 0.024; Cohen's d = 0.878, large effect size; permutation test, p = 0.018; t(26) = 2.51, p = 0.018; Cohen's d = 0.924, large effect size	DS < FS
91-102 ms	Left precentral gyrus (6L; -60, -5, 35)	0.102 ± 0.25 (DS) 0.516 ± 0.61 (FS)	permutation test, p = 0.031; t(26)= 2.34, p = 0.028; Cohen's d = 0.862, large effect size;	DS < FS
99-101 ms	Left inferior parietal lobule (40L; -65, -30, 40)	0.067 ± 0.23 (DS) 0.368 ± 0.44 (FS)	permutation test, p = 0.039; t(26) = 2.27, p = 0.032; Cohen's d = 0.832, large effect size	DS < FS

149-152 ms	Right superior parietal lobule (7R; 30, -70, 55)	0.05 ± 0.24 (DS) 0.311 ± 0.34 (FS)	permutation test, p = 0.029; t(26) = 2.29, p = 0.030; Cohen's d = 0.865, large effect size	DS < FS
263-268 ms **273-280 ms**	Right superior temporal gyrus (22R; 65, -15, 5)	0.176 ± 0.19 (DS) 0.037 ± 0.12 (FS); 0.204 ± 0.19 (DS) 0.055 ± 0.12 (FS)	permutation test, p = 0.023; t(26) = 2.39, p = 0.024; Cohen's d = 0.906, large effect size; permutation test, p = 0.016; t(26) = 2.51, p = 0.018; Cohen's d = = 0.956, large effect size	DS > FS
265-268 ms **274-277 ms**	Right postcentral gyrus (43R; 65, -15, 15)	0.186 ± 0.19 (DS) 0.049 ± 0.11 (FS); 0.202 ± 0.2 (DS) 0.052 ± 0.15 (FS)	permutation test, p = 0.021; t(26) = 2.24, p = 0.034; Cohen's d = 0.904, large effect size; permutation test, p = 0.025; t(26) = 2.29, p = 0.030; Cohen's d = 0.873, large effect size	DS > FS
369-374 ms	Left middle temporal gyrus (21L; -65, -15, -5)	0.015 ± 0.04 (DS) 0.082 ± 0.07 (FS)	permutation test, p = 0.006; t(26) = 2.98, p = 0.006; Cohen's d = 1.133, large effect size	DS < FS
378-380 ms	Right middle temporal gyrus (21R; 65, -15, -5)	0.070 ± 0.09 (DS) -0.009 ± 0.08 (FS)	permutation test, p = 0.017; t(26) = 2.50, p = 0.019; Cohen's d = 0.929, large effect size	DS > FS
378-380 ms **425-427 ms**	Right superior temporal gyrus (38R; 55, 10, -15)	0.066 ± 0.09 (DS) -0.012 ± 0.08 (FS); 0.066 ± 0.04 (DS) 0.013 ± 0.05 (FS)	permutation test, p = 0.025; t(26) = 2.35, p = 0.026; Cohen's d = 0.839, large effect size permutation test, p = 0.008; t(26) = 2.85, p = 0.008; Cohen's d = 1.081, large effect size	DS > FS
454-462 ms	Right middle frontal gyrus (6R; 30, 10, 65)	0.124 ± 0.15 (DS) -0.004 ± 0.07 (FS)	permutation test, p = 0.004; t(26)= 2.71, p = 0.012; Cohen's d = 1.132, large effect size	DS > FS
456-463 ms	Superior frontal gyrus (6; 0, 6, 66)	0.128 ± 0.17 (DS) -0.02 ± 0.1 (FS)	permutation test, p = 0.005; t(26)= 2.83, p = 0.009; Cohen's d = 1.081, large effect size	DS > FS

Table 7. ROIs analysis - significant differences between DS and FS Data are represented reporting mean ± standard deviation. Trends toward significance are reported in *italic*

4.3.6 Correlations

A positive relation was evident between the activity of the ROIs centered in the left precentral gyrus (BA 6), the left inferior parietal lobule (BA 40), and stuttering severity at approximately 100 ms after TMS delivery, suggesting an increased neural effort related to a more severe disturbance. Stuttering severity was also positively correlated to the neural activity recorded in brain regions close to the right temporal cortex (BAs 22 and 43; around 265 ms after TMS) and in the superior frontal gyrus (BA 6; around 460 ms after TMS), suggesting a likely compensatory role. Stuttering severity was negatively related to the neural activity of the left temporal cortex (BA 21), at around 370 ms after TMS. Interestingly, in DS, neural activity recorded in the region surrounding the right superior temporal gyrus (BA 22 and BA 43), at 275-280 ms after TMS, was positively related with activations observed in the SMA "complex" (BA 6), at around 460 ms (r = 0.65 and r = 0.70).

Findings are summarized in Table 8.

ROIs/SSI-4	**SSI-4 spontaneous speech task score**	**SSI-4 reading task score**	**SSI-4 total score**
Mean neural activity			
Left inferior parietal lobule (BA 40) (99-101 ms)	-	-	$r = 0.77$
Right postcentral gyrus (BA 43) (265-268 ms)	$r = 0.66$	-	-
Left middle temporal gyrus (BA 21) (369-374 ms)	$r = -0.75$	$r = -0.60$	-
Time frame-by-time frame			
Left precentral gyrus (BA 6) (100-102 ms)	from $r = 0.56$ to $r = 0.70$	from $r = 0.57$ to $r = 0.74$	-
Right postcentral gyrus (BA 43) (~ 265 ms)	from $r = 0.58$ to $r = 0.77$	from $r = 0.58$ to $r = 0.72$	-
Right superior temporal gyrus (BA 22) (~ 265 ms)	from $r = 0.57$ to $r = 0.72$	from $r = 0.56$ to $r = 0.68$	-
Superior frontal gyrus (BA 6) (~ 460 ms)	from $r = 0.56$ to $r = 0.62$	-	-

Table 8. Correlations among ROIs and stuttering severity indexes.

4.4 Discussion of findings

In the present study the powerful combination of transcranial magnetic stimulation and electroencephalography allowed to shed light on the neurophysiological temporal dynamics that follow the activation of the supplementary motor cortex in adults with persistent DS. The comparison of neural sources reconstruction of TEPs in the DS group and in fluently speaking controls revealed different patterns of neural activations in terms of time and brain structures. In general, FS resulted in wider and more diffuse activations of neural networks that may be functionally connected to the SMA complex, suggesting a more distributed and efficient elaboration of the related neural signals. The DS group resulted in lower reactivity of the stimulated cortex about 65-80 ms after the TMS followed by a lower neural activity of left hemisphere speech/motor planning regions and left inferior parietal lobule at about 90-100 ms after the stimulus. From this moment on, abnormal recruitment of right hemisphere brain structures was evident. Specifically, DS participants abnormally activated the right temporal cortex in two consequent time windows between 260 and 460 ms after the TMS. Finally, at about 460 ms, they abnormally recruited the right premotor cortex and the right motor regions close to the stimulated SMA complex.

Present results highlight that in DS the lower/delayed activation of the SMA complex may negatively influence the proper activation and communication of networks involved in speech processing and in general motor behavior. The SMA complex is a central hub in motor programming and in speech production since it is strongly connected with left inferior frontal regions (Catani *et al*., 2012), with cortical and subcortical motor structures, and with cognitive and associative brain areas (Nachev *et al*., 2008). An efficient neural exchange between these structures is crucial for the generation of skilled motor acts such as the production of fluent speech. Therefore the SMA complex may be a central node in the pathophysiological mechanism of DS since it integrates the neural signals from and to a series of dysfunctional brain networks. The SMA complex manages the temporal organization of volitional, complex, motor sequences (Coull *et al*., 2015; Cona and Semenza, 2017) such as speech and the update of motor programs in consecutive movements (Shima *et al*., 1996). Herein, the lower/delayed reactivity of the SMA complex highlights that in people with DS a defective activation of structures useful to manage internally driven motor sequences may lead to abnormal neural exchange with interconnected structures and also to an insufficient or atypical movement initiation.

Interestingly, similar under activations in left premotor/inferior frontal regions and left parietal cortex immediately follow the defective activation of the SMA. The left inferior frontal cortex is involved in the control of speech and motor rhythmic skills (Hickok *et al.*, 2009) and it is connected with the SMA through the FAT (Catani *et al*., 2012), a white matter bundle whose integrity is impaired in children (Misaghi *et al*., 2018) and adults (Kronfeld Duenias *et al*., 2016) with DS. FAT connects the posterior Broca's region with proper SMA and pre-SMA (Catani *et al*., 2012) and it is involved in planning, timing, and coordination of motor sequences useful for speech initiation (Dick *et al*., 2019). Reduced functional activations of left inferior frontal structures are often reported in DS during various speech tasks (Fox *et al*., 1996; Watkins *et al*., 2008). In addition, decreased resting-state connectivity (Lu *et al*., 2012) and lower grey matter volume (Chang *et al*., 2008; Beal *et al*., 2013; Garnett *et al*., 2018) may be evident. Interestingly, brain activity in these areas may raise to normal levels when the timing of speech movements is paced to an external rhythm such as during choral speech (Fox *et al*., 1996) and it is associated with the improvement of fluency.

As already stated, the SMA complex is involved along with basal ganglia in the generation of internally paced motor sequences and together they form the "internal timing network". On the other hand, the presentation of an external rhythm favors the intervention of an "external timing network" which includes the cerebellum, the premotor cortex, and the right inferior frontal gyrus and may provide the neural substrate for the timing compensation in DS individuals (Etchell *et al*., 2014). In this light, present results suggest that abnormal under-activations of the left hemisphere may be causally related to the deficient activation of structures of the internal timing network as well as to the presence of impaired white matter connectivity in these networks (Sommer *et al*., 2002; Watkins *et al*., 2008; Etchell *et al*., 2018). The lower reactivity of the SMA complex may drive an abnormal neural exchange through the FAT and other impaired white matter connections thus resulting in insufficient activation of premotor and inferior frontal regions of the left hemisphere. Here, this is followed from about 250 ms after the magnetic stimulus, by the overactivations in right temporal regions, and finally, at about 460 ms after TMS, by the overactivity in the right dorsal premotor cortex and in the stimulated SMA complex. Structural and functional abnormalities are often reported in DS also in these regions (Etchell *et al.*, 2018). For example, increased brain volume may be evident in the right inferior frontal gyrus, in the right superior temporal gyrus, and in sensorimotor regions (Jäncke *et al*., 2004, Beal *et al*., 2007; Kikuchi *et al*., 2011). Enhanced activity of the right

hemisphere is often reported in DS during various speech tasks (Fox *et al*., 1996; Braun *et al*., 1997). This abnormal pattern of activation probably reflects compensatory mechanisms played by right motor, premotor, frontal, and rolandic opercular regions trying to overcome the impairments of the homologous regions of the left hemisphere (Neumann *et al*., 2003; Neumann *et al*., 2005; Kell *et al*., 2009). In support of this theory, right hemisphere functional and structural abnormalities are more evident in adults with DS than in children with DS (Chang *et al*., 2008; Beal *et al*., 2013) and therefore may not represent a primary cause of the disorder but may rather reflect years of plastic modifications in the attempt to counteract ineffective neural activity. The here reported spatio-temporal dynamics of brain activations support this vision. Indeed, the right neural system of people with DS seems to "react" to the lower left hemispheric activity in order to manage the preparation and release of the internally generated deficient motor program. Interestingly, this attempt is not concomitant with left hemisphere under-activations but occurs about 200-300 ms later resulting in a "neural delay" and offering the basis for the occurrence of blocks and repetitions.

In conclusion, present results suggest that in adults with DS the dysfunctional activation of the SMA complex may contribute to a delayed and/or insufficient activation of structures of the left hemisphere involved in the management of speech motor acts thus favoring the impaired generation of speech gestures. This deficient pattern of activity is followed by an abnormal "reaction" of right temporal/motor structures, that may try to compensate for the defective activity of the left hemisphere and result in abnormal spatio-temporal neural dynamics. This picture is confirmed also by significant positive and negative correlations in neural activity in DS, and between ROIs and indexes of stuttering severity. Additionally, it is fully compatible with the evidence suggesting that DS may be related to weakened white matter connections especially in the left hemisphere (Kronfeld-Duenias *et al.*, 2016; Etchell *et al*., 2018) and between different networks involved in speech motor control and to compensatory attempts of the neural system to overcame neural deficits. Present results highlight that abnormal brain activations in DS are related to the deficient activation of the supplementary motor complex: abnormal white matter integrity and cortico-basal-thalamo-cortical functioning may have a causal role in this evidence, also supporting the theory that DS is a more general motor timing disorder (not only restricted to speech, considering that present findings have been obtained in absence of a speech task), in which impaired communication within and between large neural networks may be fundamental.

5 Study 2

5.1 Introduction

The multimodal investigation of the neural system of people with DS has consistently highlighted the strong involvement of the cortico-basal-thalamo-cortical networks in the disturbance (see Alm, 2004a; Etchell *et al*., 2018). These networks are mainly composed of associative cortical motor regions and basal ganglia, in a mutually interconnected way (see Alm, 2004a for a perspective in DS). The motor loops of these networks are involved in the preparation and control of internally driven motor sequences such as speech. Indeed, they promote the generation of self-paced intended motor programs and, at the same time, prevent the activation of competing motor pattern generators (Mink, 2018). Thus, the execution of a skilled motor act requires the cortico-basal-thalamo-cortical networks to correctly modulate the neural activity of primary motor cortex representations of all the muscles that may be potentially involved in the desired motor sequence (Calabresi *et al*., 2014). In this context, defective cortical excitability of both speech-related (Busan *et al*., 2016) and not speech-related (Sommer *et al*., 2003; Busan *et al*., 2013) muscles and abnormal modulation of intracortical motor circuits (Neef *et al*., 2011a; Busan *et al*., 2016) are often reported at rest in adults with DS. Compatibly, the balance between excitatory and inhibitory neural signals to the primary motor cortex may further modulate the correct implementation of complex motor sequences and thus the mutual influence between different motor effectors. Interestingly, this mechanism is impaired in many basal ganglia related motor disorders such as Parkinson's disease and focal dystonia in which the primary motor cortex of muscles that are not directly involved in desired motor acts may show abnormal functioning of intracortical networks useful to modulate the correct implementation of the final motor output (Sohn and Hallett, 2004; Shin *et al*., 2007).

As a consequence, the present study aims to investigate the functioning of the interplay between surrounding muscular effectors in order to test the hypothesis that developmental stuttering may be associated with abnormal modulation of intracortical inhibitory mechanisms of muscles not directly involved in the desired motor acts. To achieve these objectives single and paired pulse transcranial magnetic stimulation have been used to assess cortical excitability and intracortical functioning of primary motor cortex representations of hand muscles potentially but not actually involved in a simple motor act but potentially recruited for successive, related movements. Motor evoked potentials

were bilaterally obtained at rest, during a sustained motor contraction, as well as during internally paced and externally cued phasic movements.

5.2 Materials and methods

5.2.1 Participants

Thirty-one right-handed male adults were recruited for this study. Fifteen (age range 24-47 years, mean 32.3, standard deviation [SD] ± 8.0) reported a history of DS since childhood (DS group) while the other sixteen (age range 21-48 years, mean 29.8, SD ± 7.4) were fluent speakers (FS group) with no self-reported history of stuttering or other speech disorders. Three participants (1 DS; 2 FS) dropped out in the preliminary stages of the study (mainly due to TMS discomfort in the initial phases of the experiment) and were not included in the analysis. Fourteen DS participants (age range 24-47 years) and fourteen FS participants (age range 21-48 years) completed the experimental procedures. Technical problems limited data acquisition in 1 DS participant. Groups were matched for variables such as age, handedness, smoking habits, level of education, amount of musical and sports training, migraine diagnosis, and presence of depressive symptoms. All participants were Italian native speakers and none of them reported a history of major neurological disorders, psychiatric disorders, or severe brain injuries. None of the participants showed neurological abnormalities (other than stuttering in the DS group), was under pharmacological treatment with psychiatric medications or used to assume psychoactive drugs at the time of the study.

Participants gave a written consent and authorized the use and process of personal data in compliance with the Italian Law. The experimental procedure was approved by the Local Ethics Committee and was in accordance with the "World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects" and recent TMS guidelines (Rossi *et al*., 2009; Rossi *et al*., 2021).

Participants were allowed to leave the experimental procedures in any moment without giving reasons to researchers and did not receive any compensation for participating in this study.

Table 9 summarizes the demographic characteristics of participants, in both groups.

Characteristics/Groups	DS	FS	p-value	p-value (TMS group)
Age	32.3 ±8.0	29.8 ±7.4	0.38	0.52
Education	17.2 ± 3.6	15.8 ± 2.1	0.33	0.11
Handedness	85.4 ± 12.0	85.2 ±12.2	0.82	0.69
Smoke habits	4/11 (4/10)	4/12 (4/10)	0.76	1
Migraine	2/13 (2/12)	1/15 (1/13)	0.95	1
Musical training	5/10 (5/9)	4/12 (4/10)	0.91	1
Physical training	7/8 (7/7)	13/3 (11/3)	0.10	0.24

Table 9. **Main characteristics of participants**. Data are represented reporting mean ± standard deviation. (TMS group in brackets)

5.2.2 Behavioral/cognitive assessment

Handedness was assessed through the Edinburgh Handedness Inventory (Oldfield, 1971). Cognitive and behavioral evaluation was assessed by the administration of the Italian adaptation of the Beck Depression Inventory-II (BDI-II; Beck *et al*.,1996). Speech attitudes were evaluated by administering the Italian adaptation of the adult form of the Communication Attitude Test (BigCAT; Vanryckeghem and Brutten, 2012).

Stuttering severity was assessed through the Stuttering Severity Instrument-4 (SSI-4; Riley *et al.*, 2009) amongst DS group members only. DS participants were audio-video recorded during about 3-5 minutes of spontaneous speech and a reading task of the same written text. Stuttering severity was assessed in terms of frequency, duration, physical concomitants, and naturalness of the individual's speech. Fluently speaking controls were evaluated by a trained researcher before the beginning of the experimental procedures to exclude the presence of undetected stuttering or other speech disorders.

5.2.3 Transcranial Magnetic Stimulation setting

Participants sat on a comfortable chair for the entire duration of the experimental procedures and wore a lycra cap on which a grid of 10 mm spaced point was drawn in order to better individuate the region on the scalp that allowed to obtain the most evident and reliable motor evoked potentials (MEPs). Self-adhesive disposable electrodes (Ag/AgCl) were bilaterally placed on a tendon belly montage over the *first dorsal interosseous* (FDI) muscle and over the *abductor digit minimi* (ADM) muscle. A ground electrode was placed on the right forearm. TMS (Medtronic MagPro R30) biphasic stimuli

were administered by applying a “figure-of-eight” stimulation coil (Medtronic C-B60 – wing outer diameter about 75mm; antero-posterior direction of the first phase of the current in the coil) on the participant’s scalp at the primary motor cortex level. During the stimulations, the coil was manually positioned and maintained on the scalp with the handle pointing backwards at a 45° angle with respect to the medial longitudinal fissure. Eelectromyographic (EMG) activity was recorded using a digital band-pass filtering of 20-2000 Hz (sampling rate 8000 Hz). Participants were asked to seat in a totally relaxed position with elbows flexed at 90°, with hands pronated on their legs, and to keep their eyes open during the stimulations. The coil was positioned on the fronto-central region of the subject’s scalp and stimuli were applied to identify the best motor representation of the contralateral ADM muscle on the basis of MEPs amplitude on the EMG trace. The “hot-spot”, namely the scalp point in which the TMS induced the maximum MEP, was identified moving the coil in steps of about 10 mm. The hot-spot was marked on the cap with a piece of surgical tape to ensure the accurate positioning of the coil throughout the experiment. Resting motor threshold (RMT) was individuated as the minimum stimulation intensity resulting in a MEP of at least 50 μV in half of 8-10 consecutive trials, when stimulating the ADM hotspot. The muscular resting state was always verified by on-line visual inspection of the EMG trace. About 5 MEPs were obtained at rest stimulating the ADM hotspot at the 130% of RMT. After this procedure, participants underwent paired-pulse stimulation (conditioning stimulus delivered at 70% of RMT; test stimulus delivered at 130% of RMT) with interstimulus intervals (ISIs) set at 3 ms and 5 ms. About 5 MEPs were obtained at rest for each ISI and hemisphere. The same stimulation protocols were applied over the ADM hotspot during 3 tasks requesting the main activation of the FDI muscle:

- tonic contraction of the contralateral FDI;
- single phasic contraction of the contralateral index finger cued by an external acoustic stimulation (frequency 605 Hz; duration 230 ms);
- single phasic contraction of the contralateral index finger in a “self-paced” condition (i.e. the start of the contraction was not triggered by external stimuli but defined on a voluntary bases).

Participants were instructed to perform the tasks minimizing the activation of the ADM muscle. Conditions were randomized and about 5 MEP per condition were obtained bilaterally. TMS was manually delivered during tonic contractions. On the other hand,

TMS was delivered by a customized electronic device about 10-60ms after the passage of the index finger over an optic sensor during phasic contractions. In this way, stimulations were delivered in a time window comprised between 100 and 200 ms after the onset of the EMG activity of the moving FDI muscle. Contralateral and ipsilateral FDI and ADM EMG activity (starting from 60 ms before TMS delivery) was always recorded to verify the levels of contractions.

The experimental setting is summarized in Figure 6.

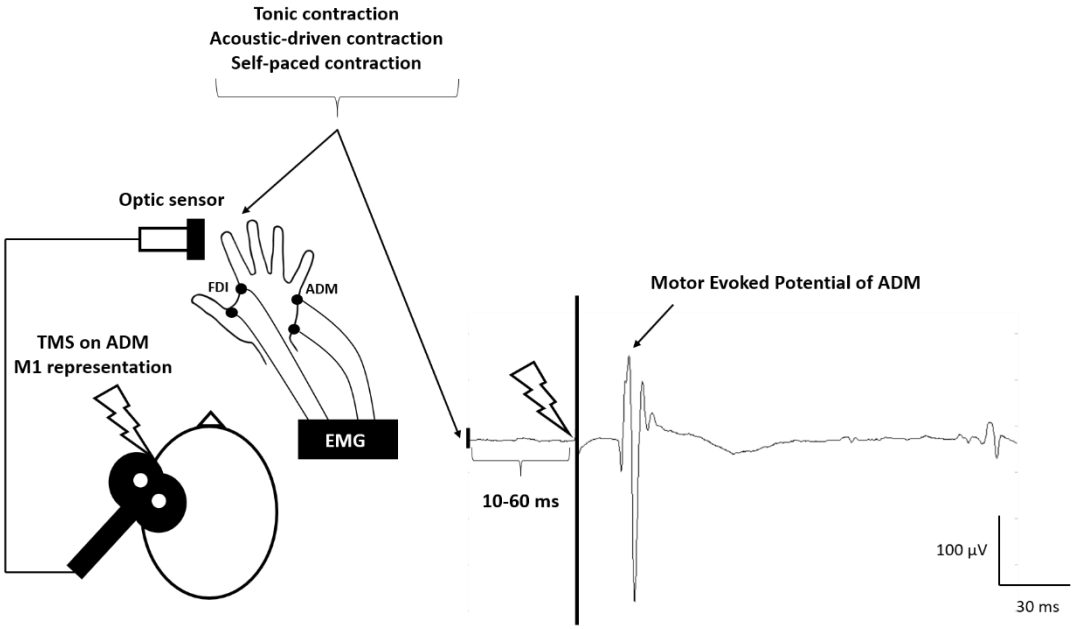


Fig. 6. **Schematic representation of the experimental setup.**

5.2.4 Data analysis

BigCAT and BDI-II were assessed by a professional psychologist. Stuttering severity was assessed considering the percentage of stuttered syllables, the length of blocks, and the severity of the secondary movements associated with stuttering during the spontaneous speech and the reading tasks. Measurements were converted to scale scores according to the SSI-4 (Riley *et al.*, 2009).

When considering TMS data, EMG traces were visually inspected. MEPs onset (first positive or negative deflection from the baseline after TMS pulse), MEPs offset (the point when EMG returns to baseline level), MEPs highest positive and negative peaks were manually defined (see Fig. 7). The RMT was expressed as the percentage of the maximum stimulation output of the TMS. MEPs peak-to-peak amplitude (µV), MEPs area (V/s), and MEPs latency (ms) were calculated. Pre-TMS EMG activity of FDI and ADM (from -60

ms) was also computed and expressed in V/s. The ratio of MEP amplitudes and areas acquired through paired-pulse protocol vs single pulse protocols was calculated to obtain indexes of intracortical inhibition of motor networks.

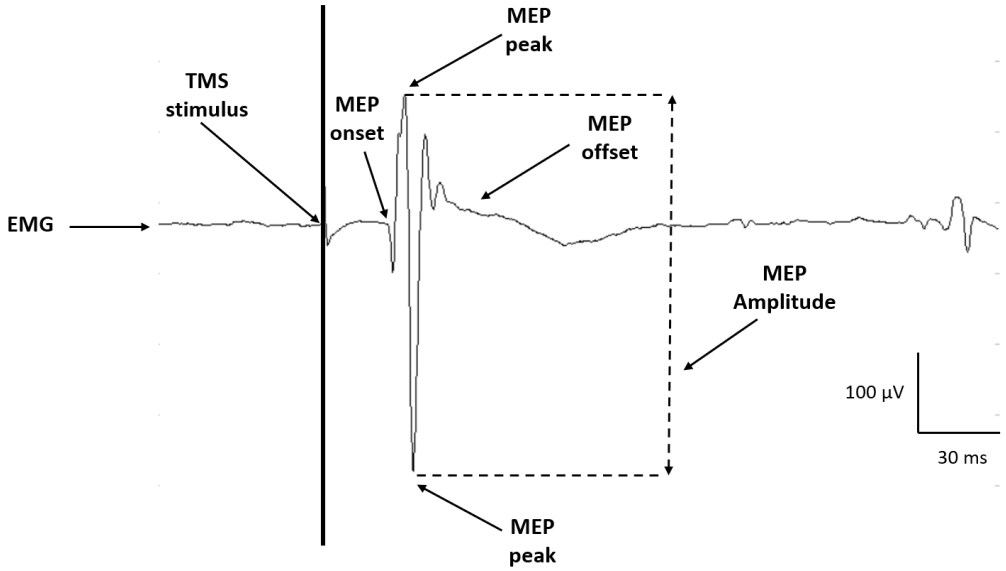


Fig. 7. **Main parameters evaluated to obtain indexes of motor evoked potentials (MEPs amplitudes, areas, and latencies).**

5.2.5 Statistical analysis

Behavioral data were compared using Student's *t*-test (normally distributed and homogenous data), Welch's *t*-test (normally distributed but not homogeneous data), Mann-Whitney non-parametric test (not normally distributed data), or Chi-square statistic (with Yates correction, categorical data). RMT, MEP data (amplitudes, areas, latencies), and pre-TMS EMG activity were analyzed using linear mixed models (West *et al*., 2006). The statistical analysis was carried out using the free statistical software R (R Core Team, 2020). RMTs were compared evaluating the effects of groups (DS vs. FS), stimulated hemispheres (left vs. right) and their interactions. MEP data of each participant were averaged for each condition. Factors analyzed were groups, stimulated hemisphere, condition (i.e. rest; tonic contraction; single phasic "acoustic driven" contraction; single phasic self-paced contraction) and their interactions. Pre-TMS EMG data of FDI and ADM were analyzed considering the effect of groups, stimulated hemisphere, side of contraction (left hand vs right hand), recording condition (i.e. tonic contraction, single

phasic “acoustic driven” contraction, and single phasic self-paced contraction), and their interactions.

A post hoc-analysis was performed depending on data characteristics using the Student’s *t*-test, Mann-Whitney test or Wilcoxon test. An estimate of the corresponding effect size was also reported when “two means” comparisons were encountered depending on data normality and the statistical design. Effects were reported in absolute values using *Hedges’ g*/*Cohen’s* $d_{unbiased}$, *d* ($0.2 < d_{unbiased}$, $d < 0.5$ = small effect; $0.5 < d_{unbiased}$, $d < 0.8$ = medium effect; $d_{unbiased}$, $d > 0.8$ = large effect), or *r* values ($0.1 < r < 0.3$ = small effect size; $0.3 < r < 0.5$ =effect size; $r > 0.5$ = large effect size). When considering non-parametric comparisons both *r* and $d_{unbiased}$ -or *d*- were reported. A $p < 0.05$ was considered significant (in this case, a p comprised between 0.05 and 0.1 was considered as a trend toward significance). Finally, correlation analysis was also performed among neurophysiological data and indexes of stuttering severity by using Pearson’s correlation for normally distributed data (Spearman’s correlation was used for not normally distributed data. Gamma correlation was used for not normally distributed data in the presence of “tied observations”). A $p < 0.05$ was considered as significant.

5.3 Results

5.3.1 Behavioral/cognitive assessment

BigCAT showed a statistically significant difference between DS and FS participants revealing a negative attitude toward speech and speech abilities in the DS group ($p<0.001$; see Table 10).

Stuttering severity was classified as very mild in two DS participants, mild in three, moderate in seven, and severe in three.

Data from BigCAT and BDI-II are summarized in Table 10.

SSI-4 scores and BigCAT scores of DS participants are summarized in Table 11.

Characteristics/Groups	**DS**	**FS**	**p-value**	**p-value (TMS group)**
BDI-II	4.2 ±4.7	2.9 ±3.9	p = 0.53	p= 0.53
BigCAT	**23 ±10**	**4.4 ±3.3**	**p < 0.001**	**p < 0.001**

Table 10. **Behavioral and cognitive profile of participants**. Data are represented reporting mean ± standard deviation. Significant differences are reported in bold.

DS Participant	SSI-4 score	Percentile	Classification	BigCAT
A	12	1-4	Very mild	7
B	13	5-11	Very mild	7
C	18	12-23	Mild	15
D	21	24-40	Mild	9
E	23	24-40	Mild	25
F	25	41-60	Moderate	32
G	25	41-60	Moderate	21
H	26	41-60	Moderate	27
I	28	61-77	Moderate	14
J	30	61-77	Moderate	32
K	31	61-77	Moderate	32
L	31	61-77	Moderate	33
M	32	78-88	Severe	32
N	32	78-88	Severe	32
O	36	89-95	Severe	27

Table 11. **Results obtained from the Stuttering Severity Instrument-4 (SSI-4) and the BigCAT scale in the DS group.**

5.3.2 Transcranial magnetic stimulation

5.3.2.1 Resting motor thresholds

Resting motor thresholds obtained stimulating the primary motor cortex representations of ADM muscles did not result in significant differences between groups (DS *vs* FS) and between stimulated hemispheres (left *vs* right). Significant differences were neither evident when considering the interaction between groups and the stimulated hemisphere. RMT findings are summarized in Table 12.

TMS index/Groups	DS		FS	
	LH	RH	LH	RH
RMT (%)	46.5 ± 10.4	49.1 ± 11.4	49.9 ± 8.5	48.7 ± 7.2

Table 12. **Resting motor thresholds.** Data are represented reporting mean ± standard deviation. LH= left hemisphere; RH= right hemisphere.

5.3.2.2 Single pulse stimulation – 130% RMT

5.3.2.2.1 MEPs peak-to-peak amplitudes

The statistical model resulted in significant differences ($p < 0.001$). An effect of the main factor related to the performed task (tonic contraction $t_{24} = 2.55$, $p = 0.018$; acoustic-driven phasic contraction $t_{24} = 3.08$, $p = 0.005$; self-paced phasic contraction $t_{24} = 2.96$, $p = 0.007$) was evident, indicating that MEPs recorded at rest were lower than those recorded when tonic and phasic movements were performed, in both groups. On the other hand, the interaction among groups, performed tasks and stimulated hemisphere resulted as significant ($t_{20} = 2.62$, $p = 0.016$). Compared to FS group, DS group was characterized by lower MEPs amplitudes after the stimulation of the left hemisphere. This was evident in MEPs recorded at rest (Mann-Whitney test, $p < 0.001$, $r = 0.608$, large effect size; *Hedges' g*/*Cohen's* $d_{unbiased} = 1.161$, large effect size), as well as during acoustic-driven (Mann-Whitney test, $p = 0.009$, $r = 0.486$, large effect size; *Hedges' g*/*Cohen's* $d_{unbiased} = 1.215$, large effect size) and self-paced ($t_{25} = 2.62$, $p = 0.015$, *Hedges' g*/*Cohen's* $d_{unbiased} = 1.021$, large effect size) phasic movements. In the FS group, the stimulation of the left hemisphere also resulted in higher MEPs amplitudes relative to their own right during acoustic-driven ($t_{13} = 4.18$, $p = 0.001$, $d = 0.945$) and self-paced ($t_{13} = 2.80$, $p = 0.015$, $d = 0.665$, medium effect size) phasic movements. The comparisons of MEPs amplitudes obtained during tonic contractions did not result in significant differences.

Main findings are reported in Fig. 8 and Table 13.

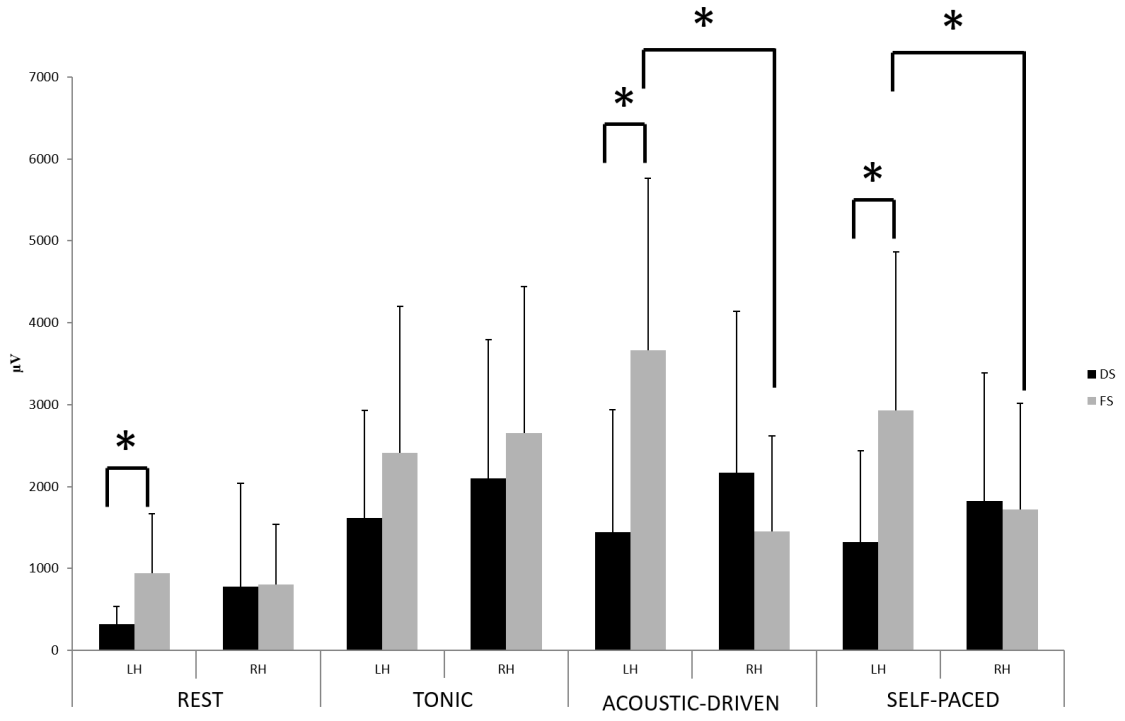


Fig. 8. **Single pulse stimulations at 130 % RMT – MEPs amplitudes.** Error bars represent standard deviation. * = significant difference; LH= left hemisphere; RH= right hemisphere.

5.3.2.2.2 MEPs areas

The statistical model resulted in a significant difference ($p < 0.001$). An effect of the main factor related to the performed task (tonic contraction $t_{24} = 2.14$, $p = 0.043$; acoustic-driven phasic contraction $t_{24} = 3.07$, $p = 0.005$; self-paced phasic contraction $t_{24} = 2.57$, $p = 0.017$) was evident, indicating that MEPs recorded at rest were lower than those recorded when tonic/phasic movements were performed in both groups.

The interaction among groups, performed tasks and stimulated hemisphere ($t_{20} = 2.94$, $p = 0.008$) resulted significant. Compared to FS group, DS were characterized by lower MEPs areas after the stimulation of the left hemisphere. This was evident at rest (Mann-Whitney test, $p < 0.001$; $r = 0.616$, large effect size; *Hedges' g*/*Cohen's* $d_{unbiased} = 1.168$, large effect size), as well as during acoustic-driven (Mann-Whitney test, $p = 0.006$; $r = 0.512$, large effect size; *Hedges' g*/*Cohen's* $d_{unbiased} = 1.184$, large effect size) and self-paced ($t_{25} = 2.84$, $p = 0.009$; *Hedges' g*/*Cohen's* $d_{unbiased} = 1.111$, large effect size) phasic movements. In the FS group, the stimulation of the left hemisphere also resulted in higher MEPs areas relative to their own right during acoustic-driven ($t_{13} = 4.26$, $p < 0.001$; $d = 0.966$, large effect size) and self-paced (Wilcoxon test, $p = 0.013$; $r = 0.663$, large effect size; $d = 0.686$, medium effect size) phasic movements. In the DS group, a tendency

towards the evidence of lower MEP areas of the left hemisphere (when compared to their right) was obtained at rest (Wilcoxon test, p = 0.074; *r* = 0.479, intermediate effect size; *d* = 0.450, small effect size). The comparison of MEPs areas obtained during tonic contractions did not result in significant differences.

Main findings are reported in Fig. 9 and Table 13.

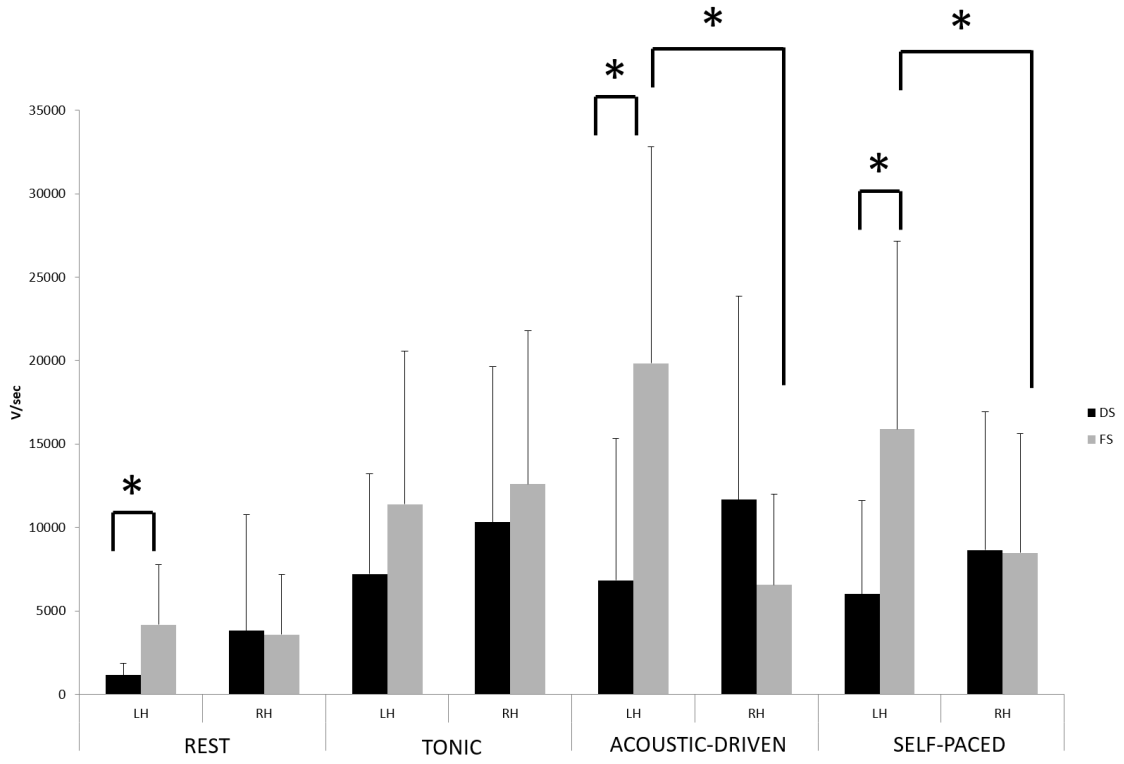


Fig. 9. **Single pulse stimulations at 130% RMT – MEPs areas.** Error bars represent standard deviation. * = significant difference; LH= left hemisphere; RH= right hemisphere.

5.3.2.2.3 MEPs latencies

The statistical model resulted as significant ($p < 0.001$) An effect related to the requested task was evident (tonic contraction $t_{24} = -4.06$, $p < 0.001$; acoustic-driven phasic contraction $t_{24} = -4.57$, $p < 0.001$; self-paced phasic contraction $t_{24} = -2.85$, $p = 0.009$) suggesting that MEPs obtained at rest were characterized by longer latencies when compared to those obtained when a contraction (tonic/phasic) was performed, in both groups.

Main findings are reported Table 13.

TMS index/Groups	DS		FS	
	LH	RH	LH	RH
Rest				
130% RMT MEPs Amplitude (μV)	**317.7 ± 222.0**	778.3 ± 1257.1	**942.0 ± 727.2**	805.0 ± 733.8
130% RMT MEPs Area (V/s)	***1152.3 ± 706.6***	*3813.9 ± 6964.3*	**4175.0 ± 3590.8**	3577.5 ± 3607.0
130% RMT MEPs Latency (ms)	22.8 ± 1.4	22.8 ± 1.5	21.7 ± 1.30	22.2 ± 1.4
Tonic contraction				
130% RMT MEPs Amplitude (μV)	1618.3 ± 1306.9	2101.3 ± 1688.6	2408.6 ± 1787.2	2653.6 ± 1784.2
130% RMT MEPs Area (V/s)	7206.7 ± 5995.2	10313.1± 9339.8	11389.4 ± 9167.9	12588.1 ± 9204.0
130% RMT MEPs Latency (ms)	21.2 ± 1.8	21.6 ± 1.5	20.7 ± 1.5	20.8 ± 1.7
"**Acoustic-driven" phasic contraction**				
130% RMT MEPs Amplitude (μV)	**1447.2 ± 1494**	2166.6 ± 1975.2	**3661.4 ± 2100.6**	**1449.9 ± 1168.1**
130% RMT MEPs Area (V/s)	**6835.6 ± 8503.1**	11672.1 ± 12203.0	**19834.3 ± 12994.4**	**6556.3 ± 5435.2**
130% RMT MEPs Latency (ms)	21.0 ± 1.3	20.9 ± 1.2	20.6 ± 1.4	20.9 ± 1.6
"Self-paced" phasic contraction				
130% RMT MEPs Amplitude (μV)	**1321.8 ± 1117.5**	1825.4 ± 1559.6	**2932.0 ± 1929.8**	**1715.3 ± 1300.0**
130% RMT MEPs Area (V/s)	**6001.7 ± 5604.7**	8647.2 ± 8284.6	**15896.0 ± 11275.5**	**8481.2 ± 7158.2**
130% RMT MEPs Latency (ms)	21.2 ± 1.2	20.9 ± 2.0	20.8 ± 1.3	21.0 ± 1.1

Table 13: Single-pulse TMS on ADM. Significant comparisons (between and within groups) are reported in bold. Tendencies toward significance are reported in italics.

5.3.2.3 Paired-pulse stimulation – ISI 3 ms

5.3.2.3.1 MEPs peak-to-peak amplitudes

The statistical model resulted as significant ($p = 0.011$). Significant differences were evident when considering the interaction between groups and the stimulated hemispheres ($t_{24} = -3.19$, $p = 0.004$). The ratio between MEPs amplitudes obtained with paired-pulse stimulation and MEP amplitudes obtained with single pulse stimulation was lower in DS (with respect to FS) when stimulating the right hemisphere (Mann-Whitney test, $p = 0.007$; $r = 0.504$, large effect size; *Hedges' g*/*Cohen's* $d_{unbiased} = 1.014$, large effect size). This evidence suggests the existence of enhanced levels of intracortical motor inhibition in DS

when the right hemisphere was stimulated independently from the experimental condition. A general tendency towards a higher intracortical inhibition of motor circuits was also evident in the left hemisphere of FS compared to their right (Wilcoxon test, p = 0.064; *r* = 0.495, medium effect size; *d* = 0.446, small effect size).
Data are summarized in Table 14.

5.3.2.3.2 MEPs areas

The statistical model resulted significant (p = 0.048). Significant differences were evident when considering the interaction between groups and stimulated hemisphere ($t_{24} = -2.86$, p = 0.009). A trend toward a significant difference was also evident when considering the interaction among groups, the stimulated hemisphere and the performed task. ($t_{20} = 1.89$, p = 0.07). The ratio between MEPs areas obtained with paired-pulse stimulations and MEPs areas obtained with single pulse TMS was lower in DS (with respect to FS) when stimulating the right hemisphere (Mann-Whitney test, p = 0.002; *r* = 0.565, large effect size; *Hedges' g*/*Cohen's* $d_{unbiased}$ = 1.112, large effect size) highlighting the existence of enhanced levels of intracortical inhibition of motor networks in DS. This was evident when a tonic contraction ($t_{26} = 2.56$, p = 0.008; *Hedges'g/Cohen's* $d_{unbiased}$ = 1.082, large effect size) and a self-paced contraction ($t_{25} = 2.69$, p = 0.012; *Hedges' g/Cohen's* $d_{unbiased}$ = 1.058, large effect size) were performed, with stronger effects during acoustic driven phasic contractions (t_{26}=4.01, p < 0.001; *Hedges' g/Cohen's* $d_{unbiased}$ = 1.630, large effect size). A tendency towards a higher intracortical inhibition was also evident in the left hemisphere of FS when compared to their right (Wilcoxon test, p = 0.056; *r* = 0.512, medium effect size; *d* = 0.465, small effect size) especially during "acoustic-driven" phasic movements (Wilcoxon test, p = 0.004; *r* = 0.512, large effect size; *d* =1.471, large effect size). Finally, higher inhibition was generally evident at rest in FS, compared to tonic and phasic movements, in both hemispheres (statistics not reported).
Data are summarized in Fig. 10 and Table 14.

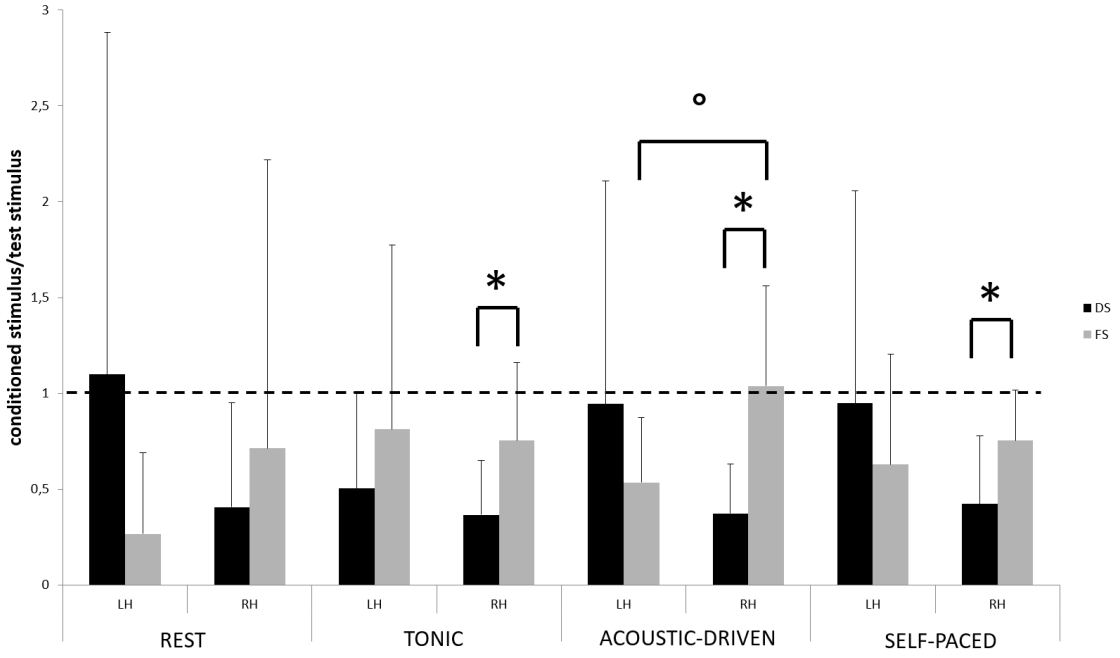


Fig. 10. **Paired-pulse protocol/single pulse protocol – ISI 3 ms – MEPs areas.** Error bars represent standard deviation. * = significant difference; ; ° = tendency toward significance LH= left hemisphere; RH= right hemisphere.

TMS index/Groups	DS		FS	
	LH	RH	LH	RH
Rest				
Paired-pulse ISI 3 ms MEPs Amplitude (ratio)	0.87 ± 1.3	0.50 ± 0.8*	0.30 ± 0.4°	0.66 ± 1.1*°
Paired-pulse ISI 3 ms MEPs Area (ratio)	1.10 ± 1.8	0.41 ± 0.5*	0.27 ± 0.4°	0.71 ± 1.5*°
Tonic contraction				
Paired-pulse ISI 3 ms MEPs Amplitude (ratio)	0.48 ± 0.43	0.39 ± 0.3*	0.75 ± 0.79°	0.74± 0.4*
Paired-pulse ISI 3 ms MEPs Area (ratio)	0.50 ± 0.50	***0.37 ± 0.3****	0.81 ± 0.96°	***0.75 ± 0.4***°**
"Acoustic-driven" phasic contraction				
Paired-pulse ISI 3 ms MEPs Amplitude (ratio)	0.86 ± 1.1	0.35 ± 0.20*	0.60 ± 0.3°	1.01 ± 0.5*°
Paired-pulse ISI 3 ms MEPs Area (ratio)	0.94 ± 1.2	***0.37 ± 0.3****	***0.53 ± 0.3***°	***1.04 ± 0.5***°**
"Self-paced" phasic contraction				
Paired-pulse ISI 3 ms MEPs Amplitude (ratio)	0.80 ± 0.7	0.43 ± 0.4*	0.73 ± 0.5°	0.77 ± 0.3*°
Paired-pulse ISI 3 ms MEPs Area (ratio)	0.95 ± 1.1	***0.42 ± 0.4****	0.63 ± 0.6°	***0.75 ± 0.3***°**

Table 14: Paired-pulse TMS on ADM – ISI 3ms. Significant comparisons are reported in bold, trends toward significance are reported in *italic*, * = Groups per stimulated hemisphere interaction, right hemisphere, DS vs. fluent speakers, ° = Groups per stimulated hemisphere interaction, left hemisphere vs. right hemisphere in fluent speakers.

5.3.2.4 Paired-pulse stimulation – ISI 5 ms

The ratio between paired-pulse stimulations delivered with an ISI of 5 ms and single pulse stimulations delivered at 130% of the RMT did not result in significant differences of main factors or interactions.

Findings are summarized in Table 15.

TMS index/Groups	DS		FS	
	LH	RH	LH	RH
Rest				
Paired-pulse ISI 5 ms MEPs Amplitude (ratio)	1.40 ± 1.8	1.71 ± 2.0	1.14 ± 1.0	1.34 ± 1.9
Paired-pulse ISI 5 ms MEPs Area (ratio)	1.71 ± 2.8	1.66 ± 2.0	1.26 ± 1.5	1.54 ± 2.5
Tonic contraction				
Paired-pulse ISI 5 ms MEPs Amplitude (ratio)	0.90 ± 0.6	0.99 ± 0.6	1.17 ± 1.0	1.21 ± 0.9
Paired-pulse ISI 5 ms MEPs Area (ratio)	0.95 ± 0.68	0.94 ± 0.6	1.27 ± 1.28	1.31 ± 1.1
"Acoustic-driven" phasic contraction				
Paired-pulse ISI 5 ms MEPs Amplitude (ratio)	1.61 ± 2.2	1.24 ± 1.0	1.02 ± 0.4	1.60 ± 1.4
Paired-pulse ISI 5 ms MEPs Area (ratio)	1.74 ± 2.2	1.26 ± 1.2	1.04 ± 0.6	1.86 ± 2.2
"Self-paced" phasic contraction				
Paired-pulse ISI 5 ms MEPs Amplitude (ratio)	1.77 ± 2.1	0.96 ± 0.8	1.42 ± 1.1	1.57 ± 1.2
Paired-pulse ISI 5 ms MEPs Area (ratio)	1.84 ± 1.8	0.99 ± 0.7	1.54 ± 1.4	1.84 ± 1.7

Table 15: Paired-pulse TMS on ADM – ISI 5ms.

5.3.2.5 Pre-TMS EMG of ADM muscles

EMG activity of ADM recorded before the TMS stimulus always resulted in higher values in the moving hand when compared to the one at rest, in both groups (TMS on the left primary motor cortex at 130% of RMT: significance of the model $p < 0.001$, main effect of the effector side $t_{26} = 3.17$, $p = 0.004$; TMS on the right primary motor cortex at 130% of RMT: significance of the model $p < 0.001$, main effect of the effector side $t_{26} = -3.50$, $p = 0.002$; paired-pulse stimulation -ISI 3 ms- on the left primary motor cortex: significance of the model $p < 0.001$, main effect of the effector side $t_{26} = 3.12$, $p = 0.004$; paired-pulse stimulation -ISI 3 ms- on the right primary motor cortex: significance of the model $p < 0.001$, main effect of the effector side $t_{26} = -3.57$, $p = 0.001$; paired-pulse stimulation -ISI 5 ms- on the left primary motor cortex: significance of the model $p < 0.001$, main effect of the effector side $t_{26} = 2.87$, $p = 0.008$; paired-pulse stimulation -ISI 5 ms- on the right primary motor cortex: significance of the model $p < 0.001$, main effect of the effector side $t_{26} = -4.20$, $p < 0.001$). Pre-TMS EMG activity recorded during paired-

pulse stimulation (ISI 3 ms ; ISI 5 ms), on the right primary motor cortex, also resulted in the trend toward significance of the interaction between groups and requested tasks (t_{23} = 2.06, p = 0.051): DS group was characterized by lower pre-TMS EMG recorded from the ADM of the left hand, during tonic contractions with respect to FS (Mann-Whitney test, p = 0.076; *r* = 0.345, medium effect size; *Hedges' g*/*Cohen's* $d_{unbiased}$ = 0.761, medium effect size). Moreover, in DS lower EGM activity was also evident in ADM during tonic contractions of the left hand when compared to "*self-paced*" phasic contractions (Wilcoxon tes t, p = 0.060; *r* = 0.523, large effect size; *d* = 1.701, large effect size). Main findings are summarized in Table 16.

EMG index (V/s)/Groups	**DS**		**FS**	
	RIGHT ADM	LEFT ADM	RIGHT ADM	LEFT ADM
Tonic contraction				
TMS at 130% RMT	543.9 ± 373.2	678.0 ± 926.6	631.9 ± 396.2	596.5 ± 551.6
Paired-pulse TMS ISI 3 ms	554.6 ± 463.9	*358.8 ± 188.6*$^{(°)(°°)}$	617.8 ± 393.5	*607.9 ± 477.3*$^{(°)}$
Paired-pulse TMS ISI 5 ms	496.8 ± 408.4	452.1 ± 179.9	668.3 ± 526.2	599.6 ± 455.9
"Acoustic-driven" phasic contraction				
TMS at 130% RMT	629.6 ± 238.8	642.6 ± 279.2	710.2 ± 352.0	644.7 ± 304.1
Paired-pulse TMS ISI 3 ms	493.1 ± 206.7	610.2 ± 591.2	738.8 ± 406.0	560.2 ± 281.5
Paired-pulse TMS ISI 5 ms	470.2 ± 150.1	638.2 ± 615.9	653.9 ± 311.7	551.3 ± 273.9
"Self-paced" phasic contraction				
TMS at 130% RMT	623.6 ± 322.4	803.0 ± 585.5	707.7 ± 308.0	618.4 ± 327.4
Paired-pulse TMS ISI 3 ms	498.9 ± 288.8	*720.5 ± 904.4*$^{(°°)}$	661.3 ± 316.3	540.3 ± 199.7
Paired-pulse TMS ISI 5 ms	543.8 ± 367.0	811.0 ± 1001.9	763.6 ± 558.9	564.7 ± 181.0

Table 16. Pre-TMS EMG in the ADM muscles. Contralateral data are reported as mean ± standard deviation. Main findings (trends toward significance) are reported in *italic*. $^{(°)}$ *groups per task interaction, DS vs. FS ;*$^{(°°)}$*groups per task interaction in DS.*

5.3.2.6 Pre-TMS EMG of FDI muscles

Pre-TMS EMG areas of the moving FDI were higher than those of the FDI at rest in both groups (TMS on the left primary motor cortex at 130% of RMT: significance of the model p < 0.001, main effect of the effector side t_{26} = 4.23, p < 0.001; TMS on the right primary motor cortex at 130% of RMT: significance of the model p < 0.001, main effect of the effector side t_{26} = -4.26, p < 0.001; paired-pulse stimulation -ISI 3 ms- on the left primary motor cortex: significance of the model p < 0.001, main effect of the effector side t_{26} = 2.74, p = 0.011; paired-pulse stimulation -ISI 3 ms- on the right primary motor cortex:

significance of the model $p < 0.001$, main effect of the effector side $t_{26} = -3.10$, $p = 0.005$; paired-pulse stimulation -ISI 5 ms- on the left primary motor cortex: significance of the model $p < 0.001$, main effect of the effector side $t_{26} = 1.71$, $p = 0.099$; paired-pulse stimulation -ISI 5 ms- on the right primary motor cortex: significance of the model $p < 0.001$, main effect of the effector side $t_{26} = -3.58$, $p = 0.001$). The comparison between groups highlighted that during the stimulation of the right hemisphere the DS group was characterized by lower pre-TMS EMG activity in the FDI muscle (TMS on the right primary motor cortex at 130% of RMT: $t_{26} = 2.19$, $p = 0.038$; paired-pulse stimulation -ISI 3 ms- on the right primary motor cortex: $t_{26} = 4.89$, $p < 0.001$; paired-pulse stimulation -ISI 5 ms- on the right primary motor cortex: $t_{26} = 5.83$, $p < 0.001$). More specifically, the interaction between groups and effector side resulted significant (TMS on the left primary motor cortex at 130% of RMT: $t_{24} = 2.57$, $p = 0.017$; paired-pulse stimulation -ISI 3 ms- on the left primary motor cortex: $t_{24} = 2.52$, $p = 0.019$; paired-pulse stimulation -ISI 3 ms- on the right primary motor cortex: $t_{24} = -3.47$, $p = 0.002$; paired-pulse stimulation -ISI 5 ms- on the left primary motor cortex: $t_{24} = 1.95$, $p = 0.063$; paired-pulse stimulation -ISI 5 ms- on the right primary motor cortex: $t_{24} = -4.27$, $p < 0.001$), highlighting that in DS lower pre-TMS EMG activity may be evident in the moving FDI muscle ($t_{26} = 2.93$, $p = 0.007$; *Hedges' g*/*Cohen's* $d_{unbiased} = 1.109$, large effect size; Mann-Whitney test $p = 0.003$; $r = 0.547$, large effect size -*Hedges' g*/*Cohen's* $d_{unbiased} = 0.925$, large effect size-; Mann-Whitney test $p = 0.001$; $r = 0.591$, large effect size -*Hedges' g*/*Cohen's* $d_{unbiased} = 1.361$, large effect size-; Mann-Whitney test, $p = 0.001$; $r = 0.591$, large effect size -*Hedges' g*/*Cohen's* $d_{unbiased} = 0.993$, large effect size-; $t_{26} = 3.05$, $p = 0.005$; *Hedges' g*/*Cohen's* $d_{unbiased} = 1.226$, large effect size). The interaction between groups and performed tasks also resulted as significant (paired-pulse stimulation -ISI 3 ms- on the right primary motor cortex: $t_{23} = -3.46$, $p = 0.002$; paired-pulse stimulation -ISI 5 ms- on the right primary motor cortex: $t_{23} = -4.95$, $p < 0.001$), as well as the effect of the interaction among groups, requested task and effector side (paired-pulse stimulation -ISI 3 ms- on the right primary motor cortex: $t_{21} = 2.35$, $p = 0.029$; paired-pulse stimulation -ISI 5 ms- on the right primary motor cortex: $t_{21} = 3.55$, $p = 0.002$). This indicates that differences between groups were evident in the pre-TMS EMG activity of the moving FDI muscle during phasic movements (lower pre-TMS EMG values in DS; post-hoc comparisons not reported; compare with Table 16). In addition, some differences were evident in the FS group when considering the performed tasks. Pre-TMS EMG areas of the left FDI muscle recorded during phasic movements were higher than those recorded during tonic contractions (paired-pulse

stimulation -ISI 3 ms-: Wilcoxon test p = 0.056; *r* = 0.512, large effect size -*d* = 0.694, medium effect size-; Wilcoxon test p = 0.003; *r* = 0.797, large effect size -*d* = 1.442, large effect size-; paired-pulse stimulation -ISI 5 ms-: Wilcoxon test p = 0.026; *r* = 0.596, large effect size -*d* = 0.747, medium effect size-; Wilcoxon test p < 0.001; *r* = 0.881, large effect size -*d* = 2.119, large effect size). Finally, higher levels of activations were always evident form a qualitative point of view, in the FDI when compared to the ADM in both groups. Higher ratios of pre-TMS EMG activity (FDI/ADM) were evident in the FS group, in every condition.

Findings are summarized in Table 17.

EMG index (V/s)/Groups	**DS**		**FS**	
	RIGHT FDI	LEFT FDI	RIGHT FDI	LEFT FDI
Tonic contraction				
TMS at 130% RMT	1426.5 ± 871.7(*)	1657.8 ± 1184.6	1763.3 ± 875.3(*)	2223.8 ± 1561.7
Paired-pulse TMS ISI 3 ms	1228.4 ± 872.0(*)	1577.3 ± 1014.2(*)	1734.3 ± 876.6(*)	***1577.9* ± *1275.6*(*)(**)**
Paired-pulse TMS ISI 5 ms	1199.4 ± 769.9(*)	1790.0 ± 852.3(*)	2330.4 ± 1186.4(*)	**1486.6 ± 1116.6**(*)(**)**
"Acoustic-driven" phasic contraction				
TMS at 130% RMT	1883.7 ± 1171.9(*)	1749.5 ± 631.7	3308.1 ± 2218.6(*)	3155.9 ± 2217.6
Paired-pulse TMS ISI 3 ms	1229.2 ± 726.4(*)	**1442.8 ± 573.2**(*)(***)**	2993.9 ± 2559.7(*)	***2999.0* ± *1927.1***(*)(**) (***)**
Paired-pulse TMS ISI 5 ms	1469.1 ± 940.3(*)	**1292.0 ± 515.8**(*)(***)**	4103.9 ± 5217.0(*)	**2670.0 ± 1712.2**(*)(**) (***)**
"Self-paced" phasic contraction				
TMS at 130% RMT	1631.5 ± 615.1(*)	1818.1 ± 742.8	3004.0 ± 1541.0(*)	2645.5 ± 1167.6
Paired-pulse TMS ISI 3 ms	1426.2 ± 715.0(*)	**1299.0 ± 537.2**(*)(***)**	3224.2 ± 3398.7(*)	**3049.8 ± 1661.4**(*)(**) (***)**
Paired-pulse TMS ISI 5 ms	1347.7 ± 627.1(*)	**1318.3 ± 445.9**(*)(***)**	3045.5 ± 2259.8(*)	**3224.9 ± 1675.8**(*)(**) (***)**

Table 17. Pre-TMS EMG in the FDI muscles. Contralateral data are reported as mean ± standard deviation. Main findings (trends toward significance) are reported in *italic*. *(*)groups per effector side interaction: DS vs. fluent speakers; TMS on the left primary motor cortex at 130% of RMT (**)groups per tasks per effector side interaction: fluent speakers, tonic contraction vs. "acoustic-driven" phasic contraction (***)groups per tasks per effector side interaction: DS vs. fluent speakers.*

5.3.2.7 Correlations

TMS parameters obtained by means of paired-pulse TMS of left and right hemisphere often positively correlated with SSI-4 scores (range from *r* = 0.41 to *r* = 0.86). In DS positive correlations were often evident among BigCAT scores and paired-pulse TMS data obtained from the stimulation of left/right primary motor cortex (ranging from *r* = 0.44 to *r* = 0.71; higher excitability resulted in higher BigCAT scores) as well as some

negative correlations (ranging from r = -0.46 to r = -0.70; higher excitability resulted in lower BigCAT scores, especially in right primary motor cortex). In DS, pre-TMS EMG areas recorded from ADM of the moving hand were positively correlated, with indexes such as BigCAT scores (ranging from r = 0.44 to r = 0.80) and SSI-4 scores (ranging from r = 0.42 to r = 0.88). This was mainly evident when considering the activity activity recorded form left hand. Positive correlations were also evident in DS between pre-TMS EMG activity recorded from the moving FDI, BigCAT scores (ranging from r = 0.43 to r = 0.74; this correlation was evident also in FS, ranging from r = 0.41 to r = 0.83) and SSI-4 (ranging from r = 0.43 to r = 0.78).

Qualitatively, TMS data obtained from the contralateral ADM muscle were more correlated in DS (12.3% of data resulted in a significant correlation), than in FS (11.7%). Similarly, TMS data were more correlated to the pre-TMS EMG data recorded from ADM muscles, in DS (5.5% of data resulted in a significant correlation), than in FS (5.1%). This was mainly evident when considering data obtained from single pulse TMS. On the opposite, TMS data obtained my means of paired-pulse protocols were qualitatively more correlated in FS. TMS data were more correlated to the pre-TMS EMG obtained from the FDI muscles of the FS group (6.1% of data resulted in a significant correlation), when compared to DS (5.3%). This was mainly evident when considering data obtained from single pulse TMS, while TMS data obtained by means of paired-pulse protocols were qualitatively more correlated in DS. Correlations among pre-TMS EMG data resulted in different patterns. Qualitative analysis revealed that ADM muscles were more correlated in DS (20.2% of data resulted in a significant correlation; 17% in FS) while the opposite was evident when considering the pre-TMS EMG data of FDI muscles (12.5% of data were correlated in DS, 16.8% in FS). Pre-TMS EMG of ADM muscles was more correlated to pre-TMS EMG of FDI in DS (11.8% of the data), than in FS (5.3%).

5.3.2.8 Control analysis

Control analyses were performed on TMS data obtained from tonic contractions and phasic movements to verify if the effects observed were specific for the ADM muscle or if they were also evident in the FDI. The experimental setup allowed to obtain and record MEPS also from the contralateral FDI when stimulation the ADM primary motor cortex representations. Single pulse protocol (130% of RMT) bilaterally resulted in lower MEP amplitudes and areas recorded from the FDI in DS (when compared to FS) especially

during phasic movements (statistics not reported). In DS, lower MEPs amplitudes/areas of the left hemisphere (when compared to the right) especially during tonic contractions (statistics not reported). As previously reported, similar findings were evident when considering the ADM muscles: as a consequence, they should be not considered as "movement"- or "muscle"-specific. When considering TMS data of the FDI obtained by means of paired-pulse protocol (ISI 3 ms). The effects were similar to those obtained from ADM (enhanced intracortical motor inhibition in DS when compared to FS), but were only evident during phasic movements. In addition, they were evident bilaterally, and resulted in lower effect sizes (left primary motor cortex, "acoustic-driven" phasic movement: peak-to-peak amplitude Mann-Whitney p = 0.012; r = 0.469, medium effect size -*Hedges' g/Cohen's* $d_{unbiased}$ = 0.141, low/no effect-; left primary motor cortex, "self-paced" phasic movement: peak-to-peak amplitude Mann-Whitney p = 0.068; r = 0.355, medium effect size -*Hedges' g/Cohen's* $d_{unbiased}$ = 0.247, small effect size-; right primary motor cortex, "acoustic-driven" phasic movement: peak-to-peak amplitude t_{26} = 2.20, p = 0.037; *Hedges' g/Cohen's* $d_{unbiased}$ = 0.856, large effect size; area t_{26} = 2.30, p = 0.030; *Hedges' g/Cohen's* $d_{unbiased}$ = 0.879, large effect size; right primary motor cortex, "self-paced" phasic movement: peak-to-peak amplitude Mann-Whitney p = 0.011; r = 0.494, medium effect size -*Hedges' g/Cohen's* $d_{unbiased}$ = 0.931, large effect size-; area Mann-Whitney p = 0.017; r = 0.464, medium effect size -*Hedges' g/Cohen's* $d_{unbiased}$ = 0.808, large effect size-). TMS data recorded form the FDI and obtained stimulating the left hemisphere showed positive correlations among MEPs data (e.g. paired-pulse data) and SSI-4 (correlations ranging from r = 0.43 to r = 0.72; similarly to ADM, lower inhibition was related to higher severity). FDI TMS data obtained stimulating the right hemisphere showed positive correlations between MEPs and SSI-4: paired and single pulse TMS data suggested that, similarly to ADM, higher excitability was related to higher stuttering severity (correlations ranging from r = 0.49 to r = 0.60). TMS data obtained stimulating the right hemisphere and recorded from the left FDI highlighted also the existence, in DS, of positive correlations among MEPs (amplitudes/areas obtained during tonic contractions at 130% of RMT, single pulse stimulations) and BigCAT scores (correlations ranging from r = 0.65 to r = 0.82)

5.4 Discussion of findings

Previous transcranial magnetic stimulation studies in DS have mainly focused on investigating the excitability of primary motor cortex representations of both speech-related and not-speech related muscles at rest (Sommer *et al*., 2003; Busan *et al*., 2013; Neef *et al*., 2011a; Busan *et al*., 2016) or during simple speech or behavioral motor tasks (Neef *et al*., 2015b; Whillier *et al*., 2018; Sommer *et al*., 2019). These studies suggest that cortico-spinal excitability and intracortical motor functioning is usually impaired in DS, especially when considering motor structures of the left hemisphere. On the other hand, the right hemisphere may be involved in attempts useful to compensate for these deficiencies. In people with DS, the defective functioning of the motor system may result in the recruitment of motor representations that are not related to the current task (Sommer *et al*., 2019). The delicate balance between neural excitation and inhibition is fundamental to shape the final level of excitability of almost the entire motor cortex and thus to drive the proper motor execution.

In the present study, TMS was employed to investigate the mutual influence between different muscular districts during movement execution in a group of adults with DS (compared to fluently speaking controls) by stimulating the primary motor cortex representations of hand muscles during a manual motor task. Cortico-spinal excitability and intracortical inhibitory functioning of muscles not directly activated in the actual motor act were studied.

These findings suggest that distinctive intracortical mechanisms of motor control may exist in adults with DS. Single-pulse stimulation of the left primary motor cortex highlighted that the excitability of corticospinal projections was significantly decreased in the DS group. MEP peak to peak amplitudes and areas were generally lower at rest and during the phasic activation of the FDI muscle. Reduced corticospinal excitability of motor structures of the left hemisphere is usually highlighted in the DS literature in both speech and not speech-related muscles (see for example Sommer *et al*., 2003; Busan *et al*., 2013; Neef *et al*., 2015b) likely in relation to abnormal basal ganglia functioning (Wu *et al.*, 1997; Alm, 2004a) and aberrant white matter connectivity of motor systems (Watkins *et al*., 2008; Connally *et al*., 2014). Conversely, intracortical motor functioning is usually normal at rest in hand motor cortices (Sommer *et al*., 2003) but an altered balance between excitatory and inhibitory neural signals may be evident (bilaterally) in primary motor cortex representations of speech-related muscles (Neef *et al*., 2011a; Busan

et al., 2016). In the present study, paired-pulse stimulation with ISI sat at 3 ms highlighted that an exaggerated level of intracortical inhibition (ICI) is present in DS in the right motor cortex of ADM during tonic and phasic contractions of the FDI muscle. Interestingly, the amount of intracortical inhibition was modulated by the requested task with maximal levels reached when phasic movements were cued by external auditory stimuli followed by volitional phasic movements and finally by tonic muscular contractions. Different patterns of intracortical inhibition may be also evident within groups: in FS, movement generation was associated with higher levels of intracortical inhibition in the left motor cortex while the opposite was evident in DS. Compatibly, the activation of muscular effectors in motor tasks that requires high levels of temporal and spatial accuracy is generally favored by the enhancement of intracortical inhibition in motor representations of surrounding muscles that do not concur in current motor execution (Stinear and Byblow, 2003). An impairment in this process may be evident in various basal ganglia related disorders such as Parkinson's disease (Shin *et al*., 2007) and focal hand dystonia (Shon and Hallett, 2004) in which the reduced intracortical inhibition in muscles not involved in the actual motor act may interfere with the release of the desired motor program. In DS, the evidence of enhanced right-hemisphere intracortical inhibition in (surrounding) primary motor cortex representations of muscles not directly involved in the requested motor act suggests that this phenomenon may be crucial in enhancing the selectivity of the desired muscular activation rather than interfering with the execution of the intended movement. More specifically, the enhancement of intracortical inhibition above normal levels during motor execution may reflect a compensatory (adaptive or maladaptive) mechanism of the right hemisphere (Preibich *et al*., 2003; Kell *et al*., 2009; Chang *et al*., 2019) useful to counteract the diffuse cortical and subcortical abnormalities (Alm , 2004a; Lu *et al*., 2010; Chang and Guenther 2020) that are evident especially in the left hemisphere in people who stutter (Sommer *et al*., 2002; Chang *et al*., 2008; Watkins *et al*., 2008). In this light, the enhanced inhibition of motor representations of muscular districts that can potentially compete with the intended ones may be fundamental in DS to ameliorate the signal-to-noise ratio of the intended motor implementation, thus preventing the generation of unwanted movement. Interestingly, in the present study, the magnitude of motor intracortical inhibition reached the maximum level when the movement was phasic and cued by an external acoustic stimulus. The generation of internally-driven and externally-paced movements rely on the activity of different brain structures that concur in the modulation of the final level of excitability of the primary

motor cortex. The elaboration of volitional internally- timed and memory-guided motor information mainly relies on the structures of the cortico-basal-thalamo-cortical network (i.e. basal ganglia and SMA – "*internal timing network*") while the neural substrate for the generation of externally-timed movements ("*external timing network*") comprise the cerebellum, the premotor cortex and the right inferior frontal gyrus (Alm, 2004a; Etchell *et al.*, 2014). As previously mentioned, the dysfunctional activity of the basal ganglia along with the consequent impairment of the thalamocortical connections to the supplementary motor complex may disrupt the correct temporal and spatial activations of motor patterns useful for the generation of internally-driven motor sequences, thus resulting in (impaired) motor programs that are based on abnormal ratios of excitatory and inhibitory neural signals (compare with *Study 1*).

In this light, DS can be considered a disturbance of the correct motor timing implementation (Ludlow and Loucks, 2003 Alm, 2004a; Civier *et al*., 2013) that is clinically evident especially during the performance of volitional and complex motor sequences such as speech. In this case, the activation of the external timing network may compensate for cortico-basal-thalamo-cortical deficits (Etchell *et al*., 2014). Dysfluencies occur mainly during self-paced speech while conditions that involve external sensory cues such as choral reading (Kalinowski and Saltuklaroglu, 2003) altered auditory feedback (Lincoln *et al*., 2006), or speaking to the peace of metronome (Brady, 1969) may transiently induce fluency in affected individuals by activating wider brain networks (Kalinowski and Saltuklaroglu, 2003). Speculatively this effect may be obtained by raising and harmonizing the signal-to-noise ratio of neural activity in affected structures, bringing it to normal levels (Toyomura *et al.*, 2011; 2015). As a consequence, present findings may be useful to shed some light on the neural (adaptive or maladaptive) compensatory mechanisms that the brain of persons who stutter may implement, trying to counteract dysfluencies and impaired mechanisms of voluntary motor programming. In addition, they suggest the existence of a neural substrate that may be exploited by common fluency-inducing techniques, to induce temporary fluency in DS.

These suggestions are confirmed when considering present pre-TMS EMG data, correlations findings, and findings obtained from control analyses. More specifically, when comparing pre-stimulus EMG activity in ADM muscles and across the different tasks (especially when stimulating the right hemisphere) internally-driven phasic movements were associated with exaggerated levels of EMG activity and thus by abnormal activation of muscles not directly involved in the actual motor act. Interestingly,

pre-stimulus EMG activation reached normal levels when phasic movements of the index finger were driven by the external acoustic stimulation thus suggesting that the exaggerated intracortical inhibition of the right hemisphere ADM representation may result in a finer neural ratio of muscular activations useful for a better management of contralateral finger (i.e. FDI) movements.

In this light, the correlation findings mainly suggest that the bilateral enhanced motor excitability may be positively related with indexes of stuttering severity. People with higher levels of dysfluencies may show higher levels of neural noise during motor performance trying to recruit more neural resources likely in a maladaptive way. This vision is confirmed when concentrating on data obtained from paired-pulse TMS (intracortical networks), that resulted in opposite qualitative findings: they seem to influence ADM activations in FS (higher correlations). In DS, higher qualitative correlations of intracortical functioning with FDI pre-TMS EMG data may indicate higher levels of interference , with respect to the correct modulation of the motor systems (on the other hand, this evidence may represent compensatory attempts). Compatibly, there were higher levels of correlations in DS when considering ADM data, and the relations between ADM and FDI data, indicating higher levels of co-activation during motor activity.

Control analysis performed on data obtained from FDI muscles revealed that mechanisms similar to those highlighted in ADM muscles may be evident. Intracortical inhibition in moving muscles (FDI) was enhanced during movement execution but in this case the phenomenon was evident bilaterally and with lower effect sizes with respect to ADM. Considering that the level of intracortical inhibition should be lower for the effectors involved in the motor performance, the augmented, bilateral intracortical inhibition of the FDI may have a pathological meaning. This is supported when considering the correlation analysis of FDI data. Patterns of motor excitability are positively related to the indexes of stuttering severity suggesting that people who stutter may try to recruit wider neural resources during movement execution, to overcome its impairments.

In conclusion, this study highlighted that in adults with DS the exaggerated right hemisphere intracortical inhibition of less involved muscles during a specific motor task may be useful to counteract typical motor deficits of motor programming that are evident in stuttering, facilitating the execution of independent movements, and thus driving the proper motor execution. In addition, the evidence of a higher modulatory effect exerted by the external acoustic stimulation on the level of intracortical inhibition, and the consequent normalization of neural activity in non-involved muscles, shed further light on

the brain dynamics behind stuttering disappearance when using common fluency inducing conditions. Overall, present results support the hypothesis that, in people with DS, dysfluencies may be only the overt symptom of a more general and subtle motor impairment (Busan *et al*., 2017). These findings may be useful in defining more effective and focused rehabilitative solutions for people with DS.

6 Study 3

6.1 Introduction

Study 1 and 2 contributed to the description and understanding of impaired neural dynamics and motor networks in developmental stuttering, concentrating on a "pure" neurophysiological perspective. Basal connectivity and simple motor tasks were used to add comprehension about this under-evaluated disturbance.

However, developmental stuttering is a "complex" motor disturbance. In fact, in people who stutter, the number and the frequency of dysfluencies and other not-speech stuttering-related behaviors may wax and wane as a function of the social situation in which they are engaged. For example, situations of social and cognitive stress, like the ones that everyone can experience when talking in front of an audience, may dramatically worsen the severity of dysfluent speech in people with DS (Steer and Johnson 1936; von Krais Porter, 1939; Shulman, 1955; Alm, 2014; Jackson *et al.,* 2016). Conversely, the severity of symptoms may tremendously decrease when the individual with DS talks in "stress-free" social situations (Steer and Johnson 1936; von Krais Porter, 1939; Van Riper and Hull, 1955; Alm, 2014). People with DS may generally show higher levels of social anxiety (Kraaimaat *et al*., 2002) and higher autonomic signs of anxiety during stressful speaking situations (Menzies *et al*., 1999). Probably, this may be the result of the negative experiences related to previous dysfluent communications (Alm, 2004b). In this light, the anticipatory anxiety, as well as negative emotions that are often experienced by people with DS in stressful social situations such as during public speaking, may be a further negative modulating factor of stuttering severity rather than a primary cause of the disturbance (Alm, 2004b). As a consequence, experiencing conditions of social pressure or a state of anticipatory anxiety in response to potentially stressful speech-related events may play a role in stuttering severity fluctuations perhaps modulating the neural and motor efficiency of speech control and programming. As previously indicated in this thesis, DS relies on a series of structural neural abnormalities (Sommer *et al*., 2002; Chang *et al*., 2008; Watkins *et al*., 2008) as well as on a deficient activity of wider motor/speech brain networks (Alm, 2004a; Chang and Guenther, 2020; Busan, 2020). Interestingly, these networks can exchange high amounts of information with a series of limbic and cognitive brain structures (such as the amygdala, the cingulate cortex, the nucleous accoumbens, the ventral tegmental areas) that are strongly involved in the management of emotional stimuli, as well as in managing "positive" and "negative" reward-related behaviors

(Busan, 2020). Quite surprisingly, the neural correlates of the speech-related stressful effects (such as speaking in front of an audience), and their influence on brain dynamics of speech preparation and production have not been yet deeply investigated, in people with DS. In fact, to the best of our knowledge, a handful of studies tried to relate affective states (e.g. anticipatory anxiety) and dysfluencies, in stuttering. For example, Toyomura *et al.* (2018) showed that adults who stutter reported a positive correlation between neural activity in the amygdala region and stuttering occurrence, during a communication speech task with a stranger. Moreover, activity in the prefrontal cortex (which may compose an emotion regulation circuitry with the amygdala), was also decreased in adults who stutter. Compatibly, Yang *et al*. (2017) mainly showed that people who stutter have increased functional connectivity of the right amygdala with the prefrontal gyrus (and the left insula) during the speech, suggesting that aberrant interactions for anxiety regulation are evident in DS, which might be responsible for higher levels of anxiety (during speech) in people who stutter.

In this context, the present study aims to shed light on the neuronal mechanisms underlying fluctuations that are evident in stuttering when comparing "more demanding" vs "less demanding" stressful (and social) situations, such as speaking in front of an audience or in an "alone" condition. This objective will be pursued by using magnetoencephalography (MEG), a neurophysiological tool that is not widely available (mainly due to its high management demands -and related costs-), but that allows to obtain data characterized by an optimal ratio of spatial and temporal resolutions. In fact, while classical neuroimaging approaches such as fMRI and PET are only informative about the location of brain activity underlying a certain behavior, they are usually not suitable to investigate rapid brain dynamics, due to their poor temporal resolution. Conversely, most neurophysiologic tools have an excellent temporal resolution but a poor spatial one (Dash *et al*., 2020). On the other hand, MEG, especially when combined with information obtained through structural MRI, can provide important information on brain dynamics and patterns with high temporal and spatial resolution. As a consequence, MEG could be an appropriate technique for measuring brain activity, especially when related to the processes useful for speech motor preparation and/or language elaboration (Walla *et al*., 2004; Hinkley *et al*., 2016).

6.2 Materials and Methods

6.2.1 Participants

Twenty-four right-handed male adults were recruited for this study. Twelve (age range 24-51 years, mean 30.9, standard deviation [SD] ± 7.8) reported a history of developmental stuttering since childhood while the remaining were twelve fluent speakers (age range 24-35 years, mean 28.3, SD ± 4.3) with no self-reported history of stuttering or other speech disorders. Participants were divided into two groups (Developmental Stuttering - DS; Fluent Speakers - FS) that were matched for variables such as handedness age, smoking habits, education, musical and sports training, and the presence of depressive symptoms. All participants were Italian native speakers. None reported a history of major neurological disorders, psychiatric disorders or severe brain injury. Moreover none of them showed neurological abnormalities, was under pharmacological treatment with psychiatric medications or used to assume psychoactive drugs at the time of the study.

The experimental procedures were carried in collaboration with the IRCCS San Camillo Hospital (Venice, Italy) where a MEG unit is available. The experimental procedures were approved by the local ethical committees and were in accordance with the "World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects". Participants were screened for evaluating possible risks related to MEG and MRI exposure.

Participants gave written informed consent and authorized the use and process of personal detail in compliance with the Italian Law no. 196 dated 30/06/2003. Participants were able to leave the experiment in any moment without giving reasons to researchers and did not receive any compensation for participating in this study.

Table 18 summarizes the demographic characteristics of participants, in both groups.

Characteristics/Groups	DS	FS	p-value
Age	30.9 ± 7.8	28.3 ± 4.3	p = 0.33
Education	16.8 ± 3.4	17.08 ± 1.7	p = 0.82
Handedness	84.7 ± 17.7	81.0 ± 12.6	p = 0.55
Smoke habits	0.38 ± 0.48	0.25 ± 0.45	p = 0.52
Musical training	0.27 ± 0.45	0.37 ± 0.47	p = 0.58
Physical training	10/2	9/3	p = 0.43

Table 18. **Main characteristics of participants**. Data are represented reporting mean ± standard deviation.

6.2.2 Behavioral/cognitive assessment

Handedness was assessed by means of the Edinburgh Handedness Inventory (Oldfield, 1971). Participants from both groups were also evaluated from a behavioral and cognitive point of view by the administration of the Beck Depression Inventory-II (BDI-II; Beck *et al.*, 1996) Speech attitudes (also evaluating speech situations, such as speaking in front of someone) were evaluated by administering the Italian adaptation of the adult form of the Communication Attitude Test (BigCAT; Vanryckeghem and Brutten, 2012). This was useful to confirm the existence of a negative feeling and attitude in the stuttering group, when facing speaking situations, especially when these are characterized by a social interaction (please see the Results section for further information).

Stuttering severity was evaluated by means of the Stuttering Severity Instrument-4 (SSI-4; Riley *et al.*, 2009) amongst the stuttering group only: participants were audio and video recorded during about 3-5 minutes of spontaneous speech and during a reading task of the same text; stuttering severity was assessed in terms of frequency, duration, physical concomitants, and naturalness of the individual's speech. Fluently speaking controls were interviewed by a trained researcher to exclude the presence of undetected stuttering or other speech disorders.

6.2.3 Experimental setup

6.2.3.1 MEG data acquisition

Before the acquisition of MEG signals, 3 ferromagnetic coils were placed on each participant's head to constantly monitor the position of the head during the experiment.

One coil was positioned about 15 mm above the nasion while the remaining two were placed at the level of the biauricular points. Ag/AgCl cup electrodes were placed on the outer canthus and on the infra-orbital ridge of the left eye to detect vertical and horizontal ocular movements (VEOG; HEOG). In addition, two electrodes were placed around the lips (orbicularis muscles) to capture the onset of EMG activity during speech production. Two additional Ag/AgCl cup electrodes were placed also on participants' chest in order to record electrocardiographic (ECG) activity. EEG activity was also recorded (data not reported in this dissertation) from 19 Ag/AgCl electrodes that were placed and equally distributed on participant's head as follows: Fp1, Fp2, F7, F8, F3, F4, Fz, Cz, C3, C4, T3, T4, T5, T6, P3, P4, Pz, O1, O2. MEG data were acquired in a shielded room with a CTF-MEG system (MISL, Vancouver, Canada).

Participants were seated on a comfortable armchair for the entire duration of data acquisition. A MEG helmet equipped with 275 gradiometers was placed around their head. Participants were asked to minimize head movements and to keep their eyes open for the entire duration of the recording session. The position of the participant's head in the helmet was continuously monitored through the CTF Continuous Head Localization System. A MEG-compatible screen was placed in front of each participant. Data were acquired using a sampling rate of 1200 Hz. The entire duration of the recording lasted about 3 hours. About 3 minutes of empty room recording (useful to define signal-to-noise ratios) were also obtained at the start and/or at the end of each recording session.

6.2.3.2 Speech production task

Participants were instructed to perform a simple reading/speech production task. A series of stimuli consisting in Italian disyllabic single words were presented on the MEG compatible screen. Participants were instructed to repeat them loudly or silently after a delayed "go" signal. Before the beginning of each block of stimuli, participants were informed if they were heard (and evaluated, in terms of dysfluencies) during the performance by an audience of five not previously known people ("audience" condition) or if no one was listening to them ("no audience" condition). In this second case, it was explained to participants that video communication with the MEG room was maintained, for safety reasons only. Participants were instructed to raise their right hand if they needed help or assistance. However, one researcher was always able to listen to the audio of the

MEG room, in order to continuously monitoring and evaluating experimental speech performances.

Each block of stimuli started with the instruction of the task to be performed (repeat aloud/remain silent) followed, after 3000-5000 ms of blank interval, by a 2500 ms presentation of the disyllabic word that participants had to see and remember. At this point after a blank interval of 3000-5000 ms, a cross signal informed the participant to perform the requested task (i.e. repeat aloud/remain silent -Fig. 11). The experiment consisted in the presentation of 4 blocks of about 70 stimuli (75% of the trials: aloud task; 25% of the trials: silence task). The order of the stimuli and conditions was randomized across participants and across blocks.

The experimental procedure was programmed with the software *Psychopy* (Peirce *et al*., 2019) and run in Python (Van Rossum and Drake 2009)

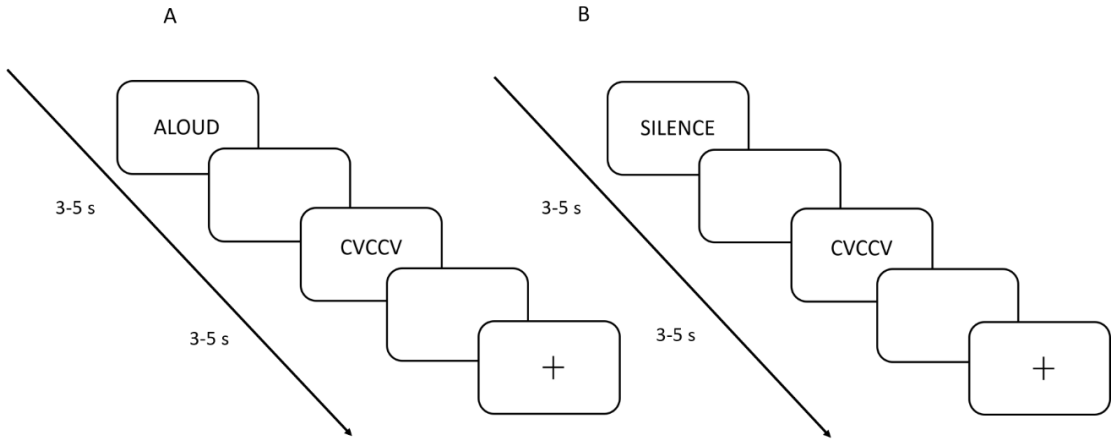


Fig. 11. **Schematic representation of the task.** Each block of stimuli consisted in the presentation of about 70 disyllabic single words that participants had to repeat loudly (A – 75% of the stimuli) or silently (B – 25% of the stimuli) after a delayed "go" signal (+).

6.2.3.3 Structural MRI acquisition

At the end of the MEG acquisition, participants underwent an MRI brain scan. Cup electrodes were removed and head localization coils were replaced with vitamin E capsules. Structural images of the brain (Fig. 12) were acquired through a 1.5 T Achieva Philips scanner (Philips Medical Systems, Best, The Netherlands) with an 8-channel head coil. Individual whole-head three-dimensional T1 –weighted TFE (TR=7.6ms, TE= 227ms, SENSE=2, FA= 8°, matrix size= 240 x 240, slice thickness = 1.2 mm, field of view = 250mm) was acquired. If a recent MRI acquisition was already available (4

participants), a precise coupling of previous images with head localization coils was realized by using a customized neuro-navigation system (Polhemus, Colchester, USA) at the end of MEG acquisitions.

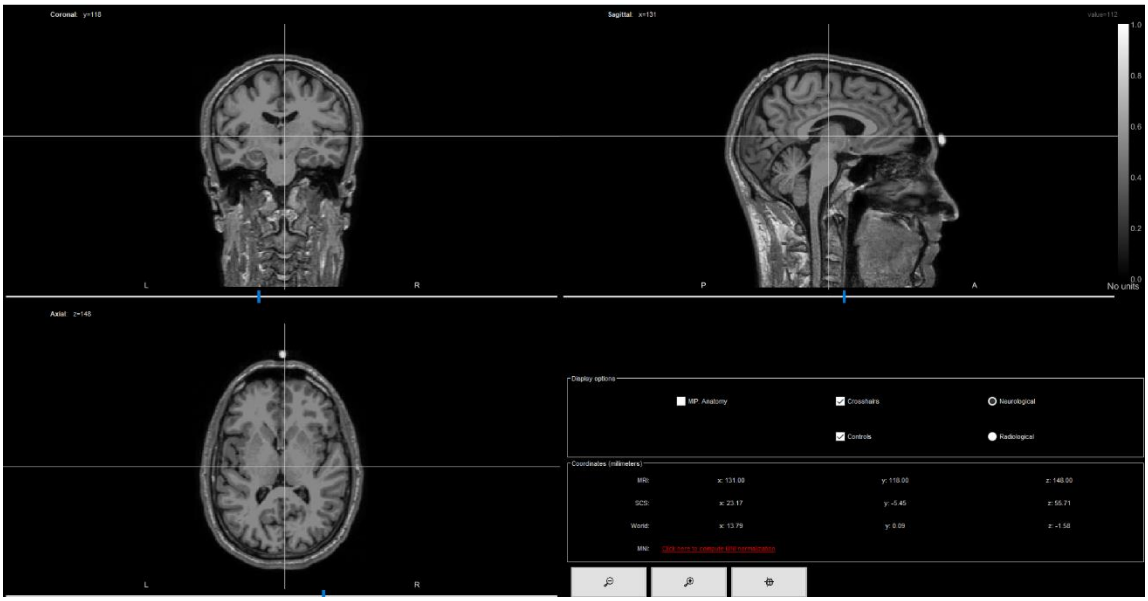


Fig. 12. Example of structural MRI of the brain obtained in a prototypical participant

6.2.4 Data analysis pipeline

MEG data were processed in MATLAB using the toolbox Brainstorm (Tadel *et al.*, 2011), which is documented and freely available for download online under the GNU general public license (http://neuroimage.usc.edu/brainstorm). The positions of the ferromagnetic coils were digitized, and MEG and MRI data were aligned. The individual cortical surface and the position of the head inside the helmet were reconstructed. Raw files were down-sampled to 600 Hz and visually inspected. EOG and ECG artifacts were automatically and manually detected and removed through Signal-Space Projection (SSP) algorithm. SSP components to be removed were always visually inspected before rejection. Raw data were further inspected and remaining segments with artifacts or noisy signals were manually removed. Power Spectrum Density allowed the identification of bad channels that were eventually removed. The accuracy of trigger markers (i.e. appearance of instructions and stimuli on the screen, appearance of the "go" signal, and start of the EMG activity of the mouth) was improved by adjusting digital triggers to the actual onset of the stimuli and "go" signals thanks to a photodiode. The EMG channel of lip muscles was

visually inspected and the onset of EMG activity related to overt speech production was manually defined. The continuous file was cut into segmented epochs. Epochs were time-locked as follows: starting from -1500 ms from the stimulus onset (appearance of the word) to +1500 ms; starting from -8500 ms from the "go" signal onset to +1500 ms; starting from -7500 ms from mouth EMG onset to +1500 ms. "Go" signal epochs and EMG epochs were longer in order to capture a reliable baseline, i.e. always before word appearance. Epochs were visually inspected and those contaminated by artifacts (except muscular artifacts related to speech production) were excluded from subsequent analysis. The inverse problem was solved using sLORETA (Pascual-Marqui, 2002) with the dipole orientation normalized to the cortical surface. Intrasubject averaging was performed for each group of epochs (i.e. word appearance, "go" signal, start of the EMG activity of the mouth) and condition ("audience"; "no audience). Finally, neural activity was re-projected to a default anatomy (ICBM152) and then spatially smoothed to allow comparisons of participants, groups, and conditions.

6.2.5 Statistical analysis

Behavioral data were compared using Student's t-test (normally distributed and homogenous data), Welch's t-test (normally distributed but not homogeneous data), Mann-Whitney non-parametric test (not normally distributed data), or Chi-square statistic (Yates correction, categorical data). Different levels of analysis were performed on MEG data (event-related fields and correspondent sources of neural activity), using Brainstorm and Fieldtrip (Oostenveld *et al*., 2011). However, considering that data analysis is still running at the time of the writing of this dissertation, only preliminary and exploratory data will be presented, with particular reference to the period of motor preparation that is evident before the start of the speech movement. This activity includes event-related fields that may be commonly referred as "motor readiness fields" typically linked to the cortical contributions of motor and pre-motor regions to the planning and preparation of voluntary and "internally generated" motor acts (Erdler *et al*., 2000). After the characterization of the correspondent event-related fields, neural source comparisons were performed considering a series of 19 regions of interest, that were bilaterally defined (Fig. 13), basing on the Desikan-Killiany surface-based anatomical atlas (Desikan *et al.,* 2006). Analyses were realized using two tailed Student's t-test on a time window comprised between -2500 ms and 0 ms before the start of the EMG activity of the mouth (i.e. before the aloud

repetition of the presented word). A level of $p < 0.01$ was considered as significant, as well as a minimum duration of significant neural activity of at least 3 ms was used to better represent plausible biological activations (see Lodish *et al.*, 2000) and further reduce possible false-positive findings.

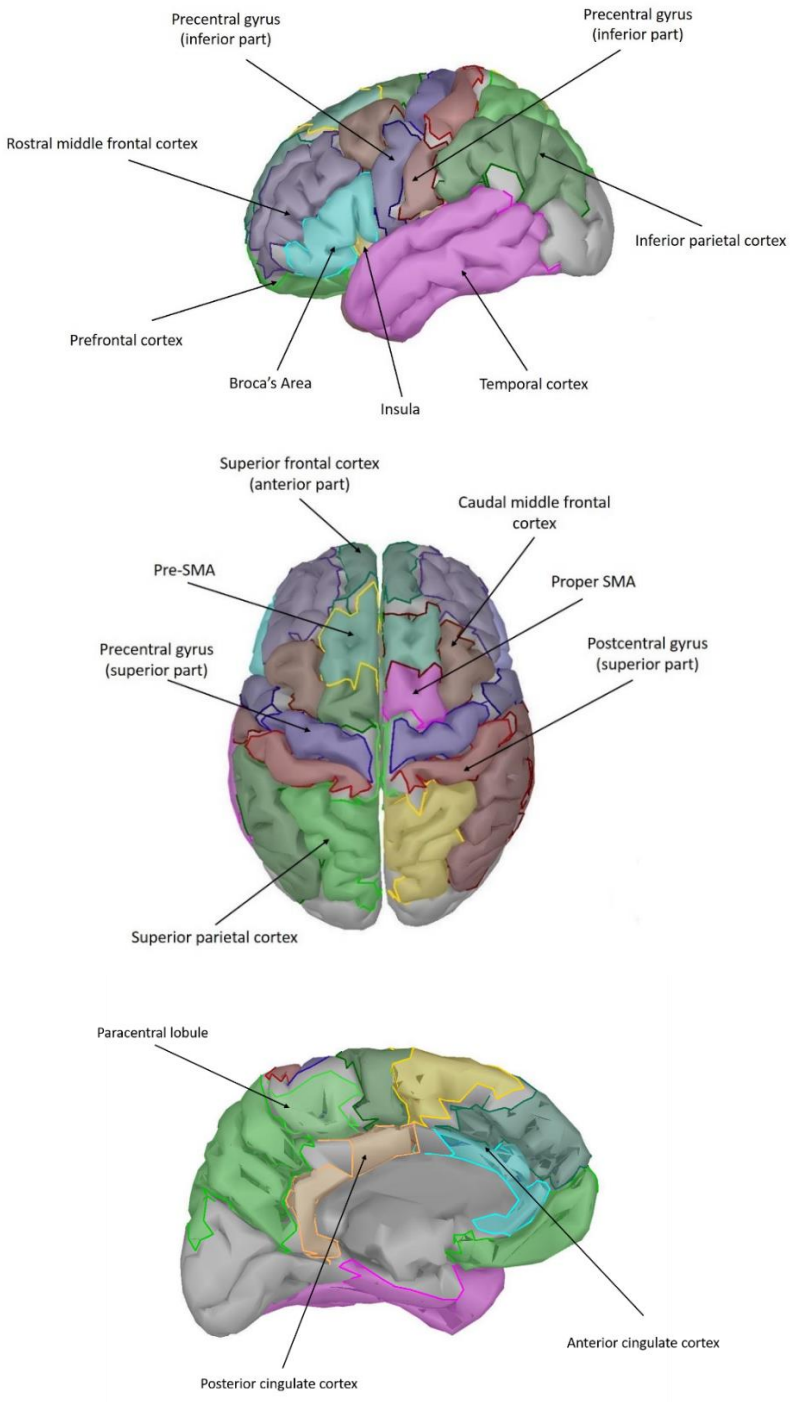


Fig. 13. Parcellation of the cortical surface in Regions of Interest based on the Desikan-Killiany atlas surface-based anatomical atlas

6.3 Results

6.3.1 Behavioral/cognitive assessment

The majority of utterances produced by DS participants were fluent. Eliciting dysfluencies in controlled laboratory settings is challenging (Sengupta *et al*., 2017) and usually, the production of single words does not evoke dysfluencies in experimental conditions (Walla *et al*., 2004; Vanhoutte *et al*., 2015).

BigCAT showed a statistically significant difference between DS and FS participants, revealing a negative attitude of people who stutter toward speech, speech situations (such as speaking in front of someone), and speech abilities (p=0.0001). This suggests that people who stutter consider dysfluencies as a limiting factor, during communication, thus resulting in higher levels of perceived difficulties.

In this context, stuttering severity was classified as mild in four DS participants, moderate in five, and severe in three.

No significant differences between groups were evident from BDI-II scale. Data from BigCAT and BDI-II are summarized in Table 19.

SSI-4 scores and BigCAT scores of DS participants are summarized in Table 20.

Characteristics/Groups	**DS**	**FS**	**p-value**
BDI-II	3.83 ± 3.6	5.25 ± 8.2	p = 0.58
BigCAT	**21.75 ±11.4**	**3.42 ± 2.7**	**p = 0.0001**

Table 19. **Behavioral/cognitive profile of participants**. Data are represented reporting mean ± standard deviation. Significant differences are reported in bold.

DS Participant	SSI-4 score	Percentile	Classification	BigCAT
A	18	12-23	Mild	24
B	19	12-23	Mild	1
C	22	24-40	Mild	29
D	23	24-40	Mild	3
E	25	41-60	Moderate	13
F	26	41-60	Moderate	28
G	26	41-60	Moderate	33
H	28	61-77	Moderate	33
I	31	61-77	Moderate	18
J	32	78-88	Severe	17
K	36	89-95	Severe	31
L	36	89-95	Severe	33

Table 20. **Results obtained from Stuttering Severity Instrument-4 (SSI-4) and BigCAT in DS group.**

6.3.2 Event-Related Fields

The visual inspection of the event-related fields (ERF) suggests the presence of higher pre-movement activity in FS in both conditions when compared to that of DS. The magnetic fields starting from about 2 sec before speech EMG onset may be compatible with the presence of a "BF". Compatibly, following the onset of speech, higher movement-evoked fields were evident in FS in both conditions when compared to that of DS.

The grand average of MEG activity for each group and condition is represented in Fig. 14, Fig. 15, Fig. 16, and Fig. 17.

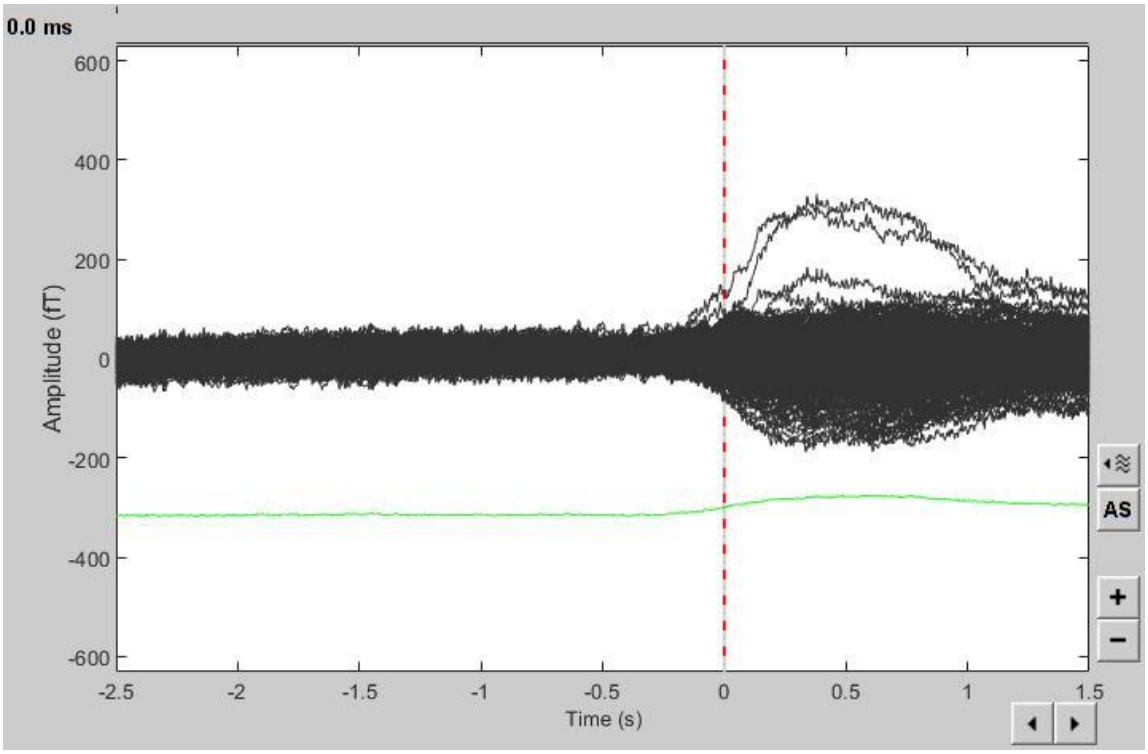


Fig. 14. **DS group – "Audience" condition.** Grand average of MEG activity time locked on the mouth EMG onset.

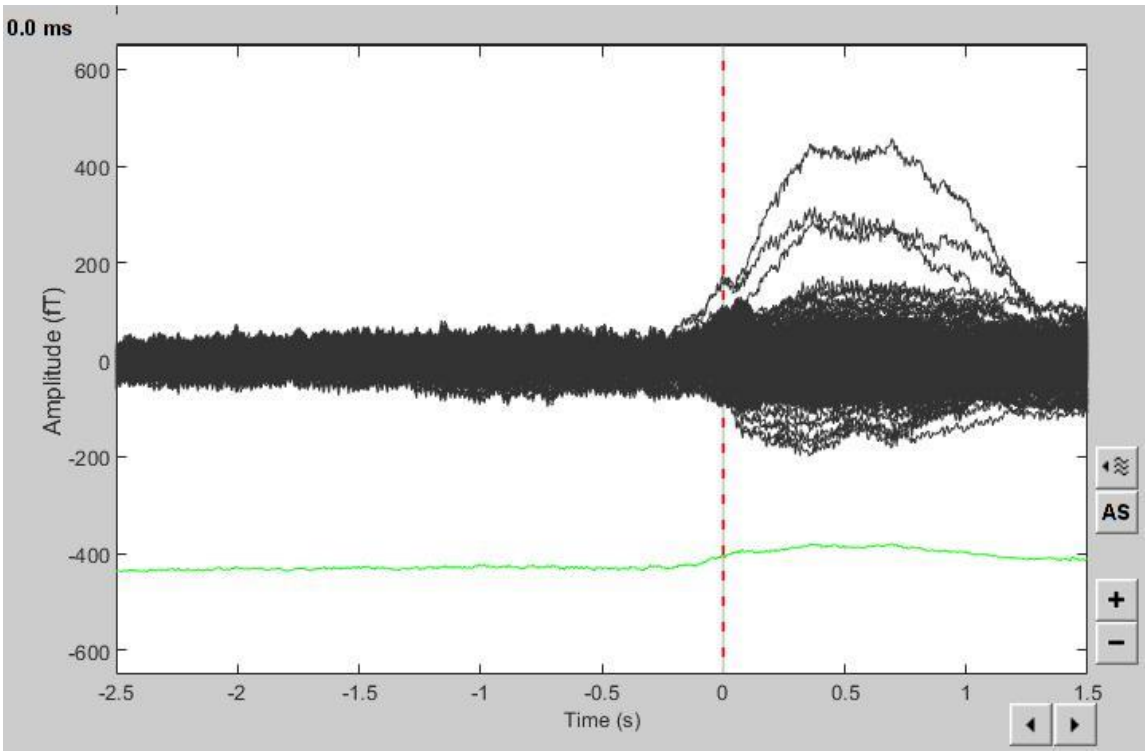


Fig. 15. **DS group – "No-audience" condition.** Grand average of MEG activity time locked on the mouth EMG onset.

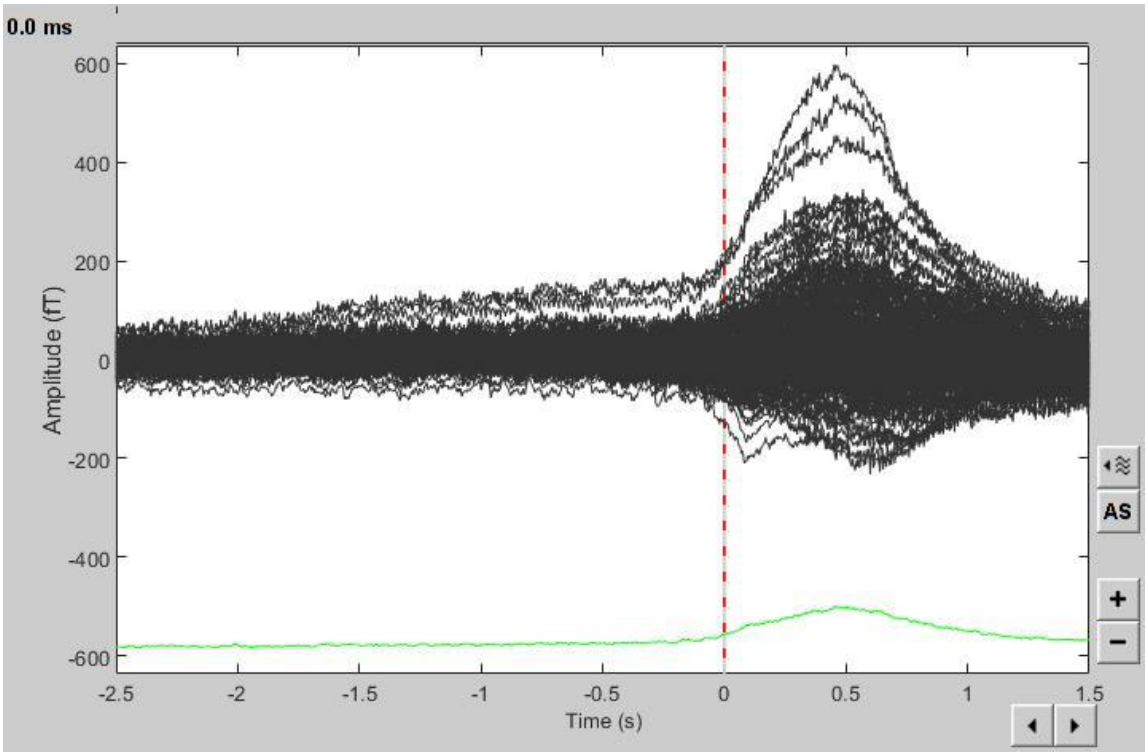


Fig. 16. **FS group – "Audience" condition.** Grand average of MEG activity time-locked on the mouth EMG onset.

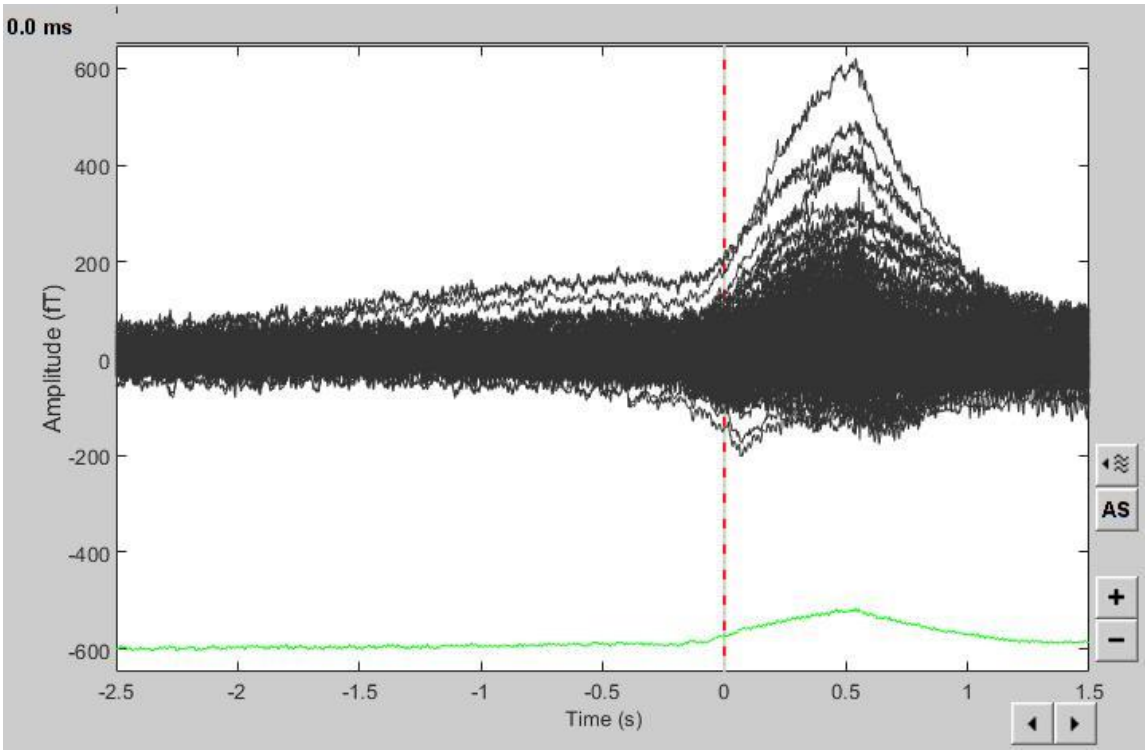


Fig. 17. **FS group – "No-audience" condition.** Grand average of MEG activity time-locked on the mouth EMG onset.

6.3.3 MEG source imaging

The comparison between groups, as well as the interaction between groups and experimental conditions, resulted in discrete patterns of significant differences ($p < 0.01$), that may be evident when considering specific regions of interest. In the following, main preliminary and exploratory findings will be reported.

6.3.3.1 "Audience" vs "No audience" in DS and FS

When considering data time-locked on mouth EMG onset, and evaluating pre-movement time-periods of motor programming (time window of interest ranging from -2500 ms to 0 ms), the contrasts between conditions highlighted discrete patterns of brain activity, in both groups.

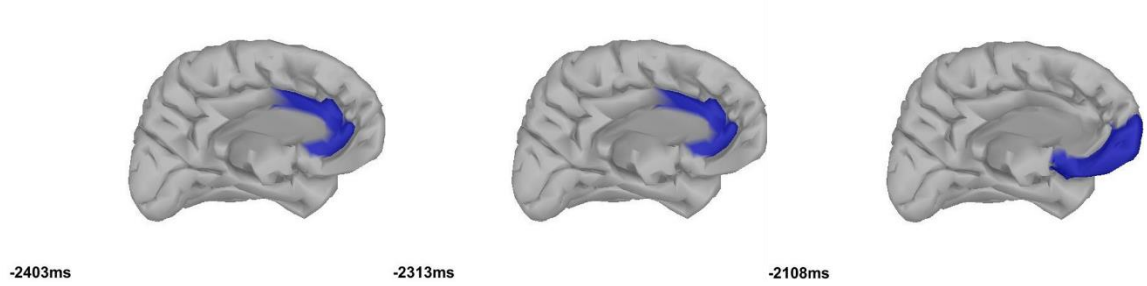
More specifically, the DS group resulted in lower activations, during the audience conditions, of the left insula (from -2500 ms to -2491ms), the left superior frontal cortex (from -2478 ms to -2470 ms), and the left anterior cingulate cortex (from -2405 ms to -2398 ms and from -2316 ms to -2306 ms). The left prefrontal cortex (from -2108 ms to -2101) also resulted in lower activity in the "audience" condition, when compared to "no audience". More interestingly, the SMA "complex" resulted as strongly and differentially involved in the task. The "audience" condition resulted in diffuse and lower activations of the right pre-SMA (from -2468 ms to -2460 ms; from -2386 to -2378; from -2228 ms to -2220 ms; from -2046 ms to -2041 ms), the left pre-SMA (from -1516 ms to -1511 ms; from -1343 ms to -1336 ms), and the left proper SMA (from -1516 ms to -1511 ms) regions.

In FS, a different and "reverse" pattern of activity was highlighted. An higher neural activity was generally more evident during "audience" condition in the left insula (from -2438 ms to -2430 ms, and from -2316 ms to -2308 ms), and in the right caudal middle frontal cortex (from -2053 ms to -2041 ms; from -2011ms to -2000 ms; from -1818 ms to -1810 ms; from -1521 ms to -1516 ms; from -1478 ms to -1470 ms; from -1408 ms to -1403 ms; from -1360 ms to -1355 ms; from -1298 ms to -1293 ms; from -1168 ms to -1163 ms). On the same line, the left pars triangularis (from -2236 ms to -2028 ms; from -1778 ms to -1745 ms; from -1656 ms to -1648 ms; from -1628 ms to -1610 ms; from -1535 ms to -1518 ms), the right prefrontal cortex (from -1858 ms to -1853 ms), the right precentral cortex (from -1853 ms to -1846 ms; from -1525 ms to -1520 ms; from -1133 ms to -1123 ms), and the left postcentral cortex (from -2013 ms to -2008 ms; from -1140

ms to -1138 ms; from -1080 ms to -1066; from -1058 ms to -1053 ms; from -1026 ms to -1018 ms; from -960 ms to -953 ms), also resulted to be more active in the "audience" condition. Finally, the right pre-SMA (from -1490 ms to -1485 ms), the left (from -1606 ms to -1596 ms; from -1533 ms to -1528 ms; from -1526 ms to -1520 ms) and right (from -1043 ms to -1038 ms) rostral middle frontal cortex, as well as the left (from -1111 ms to -1103 ms; from -1073 ms to -1070 ms; from -850 ms to -841 ms) and right (from -795 ms to -790 ms; from -416 ms to -410 ms) paracentral cortex, resulted in higher activations in the "audience" condition.

Conversely, higher neural activity of the left inferior parietal cortex (from -1987 ms to -1978 ms; from -291 ms to -286 ms; from -140 ms to -135 ms; from -131 ms to -126 ms) was found in the "no audience" condition.

Main findings (exclusively referring to the DS group) are summarized in Fig. 18 and Fig.19.



Fig, 18. DS group – "Audience" vs "No audience". Examples of activation maps for contrasts: stressor exposure ("audience" condition) resulted in lower activations (Blue: "audience" < "no audience") in the left cingulate cortex and the left prefrontal cortex, in stuttering, well before EMG activations related to speech onset.

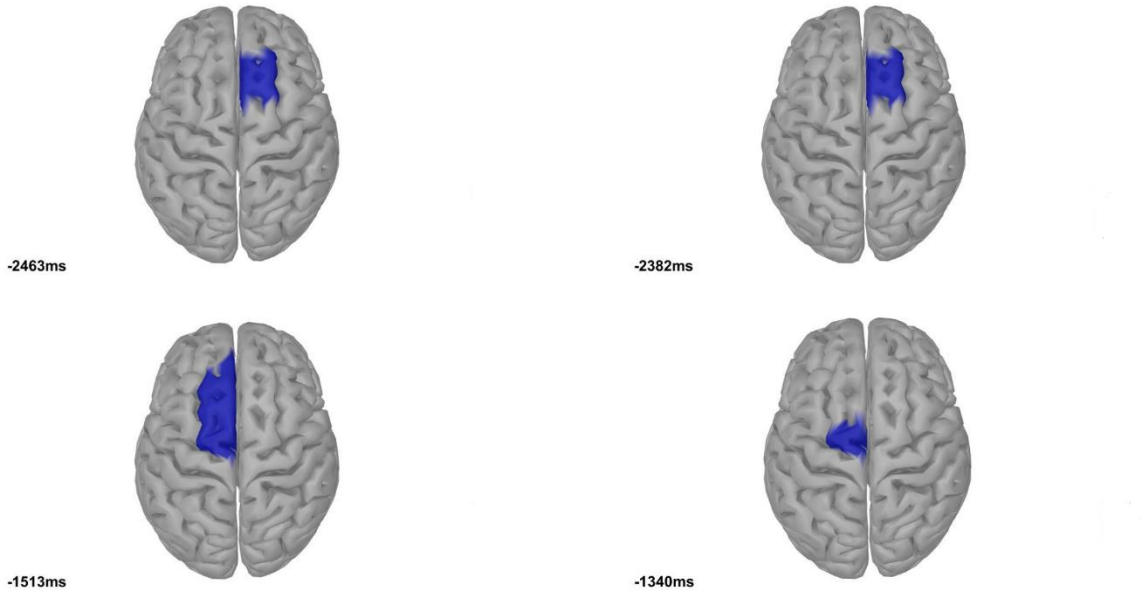


Fig. 19. **DS group – "Audience" vs "No audience".** Examples of activation maps for contrasts: stressor exposure ("audience" condition) resulted in the lower activation (Blue: "audience" < "no audience") of the right and left supplementary motor "complex", in stuttering, well before EMG activations related to speech onset.

6.3.3.2 DS group vs FS group

When comparing groups, the neural source reconstructions of the "audience" condition resulted in early and stronger neural activity, in DS, in the right postcentral cortex (from -2418 ms to -2411 ms), and the left posterior cingulate cortex (from -1173 ms to -1168 ms). No other differences reached thresholds for statistical significance.

On the other hand, when considering the "no audience" condition, higher neural activity was diffusely evident in the DS group, especially in the right hemisphere (compatibly with already available evidence in stuttering; see for example Etchell et al., 2018, for a comprehensive and recent literature revision). More specifically, higher activations were extensively detected in the right caudal middle frontal cortex (from -2125 ms to -2118 ms; from -2048 ms to -2036 ms; from -1521 ms to -1516 ms; from -1468 ms to -1456 ms; from -1370 ms to -1361 ms; form -1286 ms to -1281 ms; from -1265 ms to -1258 ms; from -1186 ms to -1180 ms; from -1168 ms to -1163ms; from 1061 ms to -1068 ms; from -945 ms to -940 ms), in the right paracentral gyrus (from -218 ms to -213 ms), the right pre-SMA (from -1208 ms to -1203 ms), and the right precentral gyrus (from -2120 ms to -2096 ms; from -1630 ms to -1625 ms; from -1168 ms to -1163 ms; from -553 ms to -545 ms; from -391 ms to -386 ms). Higher neural activations were evident also in the right

(from -2025 ms to -2020 ms; from -1911 to -1906; from -1670 ms to -1665 ms) and left posterior cingulate cortex (from -2338 ms to -2333 ms; from -2286 ms to -2283 ms; from -2031 to -2028 ms). Finally, diffuse and systematic higher activations were evident, in DS, also in the inferior parietal cortex, bilaterally, starting from about -2300 ms before starting of EMG speech activity to about -540 ms prior to speech onset.

Prototypical findings are summarized in Fig. 20 and Fig. 21.

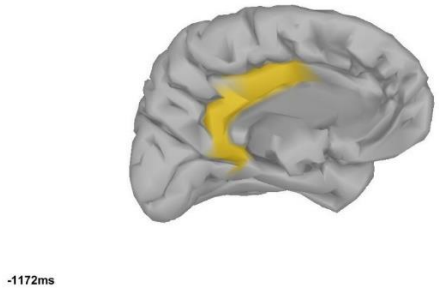


Fig. 20. **"Audience" condition – DS vs FS.** Example of activation maps for contrasts: stressor exposure ("audience" condition) resulted, in DS, in early higher neural activity of the left posterior cingulate cortex (yellow maps), well before EMG activations related to speech onset.

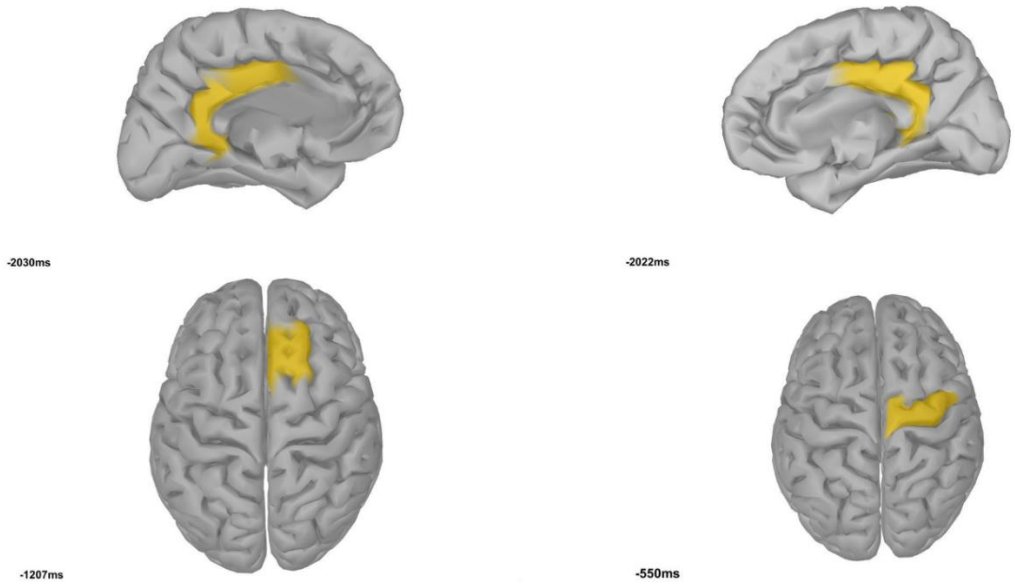


Fig. 21. **"No audience" condition – DS vs FS.** Example of activation maps for contrasts: no social stressor exposure resulted, in DS, in early and higher neural activity of brain regions such as the left and right posterior cingulate cortex (top), and (later) the right supplementary and primary motor cortices (bottom). These activities were evident well before EMG activations related to speech onset.

6.4 Discussion of findings

In the last decades, the brain functioning of people with DS has been extensively investigated at rest and during various speech and behavioral tasks (see Etchell *et al.*, 2018 for a review). However, the neural correlates of the typical stuttering severity fluctuations that can be usually observed in different "social" situations, are still obscure.

As already reported, only two previous studies have investigated the relations that may exist between anticipatory anxiety and neural activity in people who stutter. More specifically, Toyomura and colleagues (2018) revealed a positive correlation between the neural activity of the amygdala and stuttering occurrence, in adults with DS during a communication speech task with strangers. Similarly, the neural activity of the prefrontal cortex was also decreased. Interestingly, the authors suggested that the prefrontal cortex and the amygdala may be linked to form an emotion regulation circuitry (Toyomura *et al.*, 2018). In this context, Yang and colleagues (2017) showed that during speech, people who stutter have an increased functional connectivity between the right amygdala and the prefrontal gyrus (and the left insula). This suggests that aberrant interactions for anxiety regulation may be evident in DS, speculatively resulting in higher levels of anxiety during speech (Yang *et al*., 2017).

Compatibly, the present study employed magnetoencephalography to determine the neural effects that may be related to social and typically stressful situations for people who stutter, such as talking in front of a group of unfamiliar individuals. Particular attention was given to the neural dynamics related to speech preparation and production, comparing adults with DS to fluent speakers. In this context, the absence of dysfluent speech during the present tasks suggests that the negative arousal induced by the presence of an "audience" may be not sufficient to impact speech fluency of DS participants. However, this stressful social condition was sufficient to interfere with neural programming related to speech/motor preparation. In fact, exploratory analyses suggest that the social stressor exposure may result in different patterns of cerebral activations, in DS. More specifically, the comparison between conditions highlighted that the "audience" condition resulted in lower neural activity of limbic and paralimbic structures (i.e. the anterior/medial cingulate cortex and the prefrontal cortex), as well as in "de-activations" of the left and right supplementary motor cortex, well before speech production. On the other hand, speaking in front of an "audience" resulted, in FS, in higher activations of almost the entire cortical surface, including motor and speech-relevant brain areas such as the inferior frontal

regions, and the sensorimotor primary and associative cortices. This suggests that the awareness of an audience may induce in the speaker a general overactivation of brain networks, considering that a "skilled" and "public" speaking may require the recruitment of additional neural resources, to accomplish a successful performance. Some differences were evident also between groups, in both condition. More specifically, DS resulted in higher neural activations of deeper cortical regions having a potential role in attentional control, as well as in a general over-activation of cognitive and motor/speech structures of the right hemisphere, especially during "no audience" conditions.

In the following, a brief discussion of main findings will be offered, concentrating on the possible links between the limbic and motor (under)activity that has been observed in DS, during the "audience" condition. This will allow to hypothesize possible mechanisms by which the "social stress" exposure may influence speech motor programming, in people with DS.

6.4.1 The influence of emotional and attentional factors on programming of complex motor sequences (e.g. speech), in DS

Present findings suggest that, in FS, there was an higher recruitment of the left SMA complex, at about 1500 ms before speech initiation, more evident during the "audience" condition with respect to "no audience". Interestingly, a similar but opposite pattern of activations was evident in DS, in the same time window of interest. Social "stress" exposure resulted in lower activation of the SMA "complex", during the "audience" condition. In this context, this timing of activation of the SMA "complex" during speech preparation roughly overlaps the onset of the early components of the "Bereitschaftsfield" (or "readiness" field), which is the magnetic equivalent of the "Bereitschaftspotential" (or "readiness" potential; Erdler *et al*., 2000). The "Bereitschaftspotential" is a negative cortical potential, that mainly origins in the SMA "complex", well before the execution of volitional movements (about 2000-1500 ms; see Shibasaki and Hallett, 2006), including complex motor sequences, such as speech (McArdle *et al.*, 2009). Interestingly, this type of neural activity may be less evident, in people with DS, prior to speech production and also in the absence of overt dysfluent speech (Walla *et al*., 2004). Compatibly, present results suggest the presence of (qualitatively) lower activity in DS, with respect to FS, in both conditions. Interestingly, the BP is often affected also in other basal ganglia-related disorders, such as Parkinson's Disease (Shibasaki *et al*., 1978), and also in association with impaired activations of the SMA "complex" (Limousin *et al*., 1997). As previously

stated, the SMA plays a key role in speech production. The SMA "complex" is involved in the preparation of "internally-timed" motor programs (Nachev *et al*., 2008; Narayana *et al*., 2012; Etchell *et al*., 2014), and is strongly interconnected with other speech-relevant areas, such as the inferior frontal cortex (Catani *et al*., 2012). In addition, it participates in different aspects of motor and speech behaviors, ranging from lexical selection to the articulation of the final motor/speech output (Alario *et al.,* 2006). In this context, the SMA has been suggested to be part of wider and dysfunctional neural networks, that may be heavily related to stuttering: these networks may be characterized by lower/abnormal activity and exchange of information, thus resulting in lower levels of neural "synchronization", and/or higher amounts of neural "delays" (Busan *et al.,* 2019). Thus, this may easily result in motor/speech disruption and dysfluencies (e.g. Ludlow and Loucks, 2003). As a consequence, considering its role in the integration of neural signals useful for motor/speech implementation, arriving from deeper (e.g. basal ganglia, limbic and cingulate cortex), and cortical (e.g. inferior frontal gyrus, prefrontal cortex) regions, SMA may be fully considered as a key neural hub in DS (Busan, 2020). The problem seems to be that this integration is not always successful, or sufficient to activate a "fluent" speech: in fact, neural signals may arrive from a series of dysfunctional brain networks (e.g. basal ganglia, compare with *Study 2*), or, on the contrary, SMA may be not able to fully combine this information in a "fluent" motor act. Here, the reduced activity of the SMA "complex", in a time window that overlaps the "normal" Bereitschaftsfield, suggests that the exposure to a social stressor may further hamper, in DS, the programming activity of these neural structures, thus favoring the potential and successive appearance of speech dysfluencies. Interestingly, other neurophysiologic abnormalities in speech motor preparatory activity, and mainly related to the activation of the SMA "complex" and/or other structures of the CBTC network, are often reported in DS, especially before speech production (Vanhoutte *et al*., 2015, 2016; Mersov *et al*., 2016). In this context, dysfluent speech production is preceded, in people with DS, by smaller contingent negative variation (Vanhoutte *et al*., 2016), and by exaggerated beta synchronization (Mersov *et al*., 2016), thus reflecting an insufficient motor preparation and an excessively inhibited motor system. On the other hand, the slope of the contingent negative variation has been also reported to be steeper, during dysfluent speech of people who stutter (when compared to that of fluently speaking controls), and positively related to stuttering severity (Vanhoutte *et al*., 2015).

However, despite this evidence, the pathophysiological mechanisms underlying the activity of the SMA "complex", in response to a stressful social context (such as speaking in front of an audience), in DS, is not clear. Some suggestions may be inferred by previous MRI-based studies in healthy individuals (Dietrich *et al*., 2020), and in patients with social phobia (Lorberbraum *et al*., 2004). Speaking in a condition of social evaluative stress is normally associated with a peak of activation in the caudate nucleus, followed by deactivations in several brain structures involved in cognitive and emotional processing. More specifically, deactivations may be evident in the anterior cingulate cortex, prefrontal cortex, insula, putamen, and thalamus (Dietrich *et al*., 2020), but not in speech/motor areas. Compatibly, in patients with social phobia, speech anticipatory anxiety may result in greater activations of subcortical and lateral paralimbic structures including the pons, the striatum, and the amygdala as well as in lower cortical activity of the dorsal anterior cingulate and prefrontal cortex (Lorberbraum *et al*., 2004).

This evidence is well compatible with present findings, when considering that a left-lateralized deactivation of the anterior/medial cingulate cortex (AMCC) was detected, in DS, at about 2300 ms prior to speech onset. The cingulate cortex is a cortical region involved in social cognition, and in a series of cognitive processes including motivation, decision making, and error monitoring (Apps *et al*., 2016). It is an important neural hub since it is strongly interconnected with limbic structures, such as the amygdala, and the orbitofrontal cortices. Similarly, AMCC is strongly interconnected with motor structures, such as the SMA "complex" (Vogt, 2009). In this context, a lower activity of the anterior cingulate cortex may be evident in response to negative emotional stimuli, as well as in many psychiatric conditions. For example, failure of activation of the anterior cingulate cortex has been reported in patients with generalized social phobia, during emotion regulation (Blair *et al*., 2012), as well as in patients with post-traumatic stress disorder, during the recalling of negative emotional states (Lanius *et al*., 2003). Deactivation of the left anterior cingulate cortex is also evident in patients with obsessive compulsive disorder during social interactions, especially in those with higher traits of social anxiety (Ku *et al*., 2020). In the present study, the reduced activation of the left AMCC showed by people who stutter during the "audience" condition may reflect an impairment in social cognition and emotional processing of social information, i.e when speaking in front of an audience of stranger people that are evaluating their performance. This may be the consequence of a failure in a top-down control of the limbic response (Etkin *et al.*, 2011) to the stressor exposure, that, speculatively, may be the consequence of previous and negative

experiences of dysfluent speech situations, thus resulting in higher levels of social anxiety (please compare with the present BigCAT findings).

More importantly, present data suggest that the successive drop in activation of the SMA "complex" during the "audience" condition may be linked to this abnormal activity of the AMCC, in response to the stressor event. In this context, the AMCC may be an important neural link between the limbic and the motor system: strong connections are present between the SMA, the cingulate gyrus, and basal ganglia, through the medial sub-callosal fasciculus, which is also involved in the preparation/initiation of speech movements and in "emotional" aspects of spontaneous speech (Mark and Ulmer, 2002). Indeed, communication between the AMCC and the SMA complex is crucial for propositional speech (Alm, 2014): they reciprocally modulate their activity over time during the preparation of self-generated movements (such as speech), favoring the execution of the desired motor acts and contributing to the generation of the Bereitschaftspotential (Nguyen *et al*., 2014). Interestingly, Chow and Chang (2017) reported abnormalities in white matter tracts of the cingulate cortex in stuttering, in the proximity of the SMA "complex" (please compare also with Garnett *et al*., 2019). Therefore, present data suggest that, in stuttering, the lower activity of the left anterior cingulate cortex, in response to a stressful "social" condition, may contribute to negatively modulate the level of activation of the supplementary motor cortex during motor/speech preparation, thus influencing the correct motor programming and execution of planned speech movements. Finally, the here observed "negative" (i.e. lower activity in the "audience" condition, in DS) modulation of the prefrontal cortex (useful for the executive control of behaviors; ref.), in overlapping time windows of interest, may also contribute to present findings.

Some differences were evident also when comparing groups, in the various conditions. Event related fields suggest the presence of higher activity in FS with respect to DS. However, higher neural source activity was usually evident in the DS group: this may be justified considering that differences were mainly evident in regions that may be usually recruited in DS during compensation attempts, from a motor and cognitive (i.e. attentional) point of view. Compatibly higher activations of the left posterior cingulate cortex (PCC) was evident in DS, when compared to fluent speakers, in early phases of motor preparation, during both the "audience" and the "no audience" conditions. PCC is a key cortical node in the Default Mode Network (DMN – Raichle *et al*., 2001), and it is involved in a series of cognitive and behavioral processes. For example, it is highly sensitive to the arousal state and supports internally directed thoughts (Leech and Sharp,

2014). In addition, it is involved in detecting and responding to environmental events, and in controlling the balance between the internal and external attentional focus (Leech and Sharp, 2014). In DS, PCC over-activation during speech production may suggest a failure in successfully controlling the balance between internally and externally focused thoughts, with a bias towards internal sources of information. Moreover, abnormal activity within DMN nodes may play a role in DS (see Chang *et al.*, 2018), since it may interfere with the neural activity of other networks involved in goal-directed tasks, such as propositional speech (compare with Alm, 2014), thus favoring the appearance of speech disfluencies. In this context, Chang *et al*. (2018) showed that a decreased functional connectivity between the posterior cingulate cortex and regions such as the SMA "complex" may be related to stuttering persistence.

Specific patterns of higher neural activity were evident in DS (in comparison to FS) also during the "no audience" condition, especially in sensorimotor structures of the right hemisphere and, bilaterally, in superior parietal areas. Higher recruitment of the SMA "complex" was evident prior to speech production, followed by enhanced neural activity of the right primary motor cortex. Compatibly, enhanced activity of the right hemisphere is consistently reported in DS during speech tasks (e.g. Fox *et al*., 1996; Braun *et al*., 1997; Brown *et al.*, 2005; see Etchell *et al*., 2018 for a comprehensive review): this is especially evident in speech/motor areas, likely reflecting compensatory mechanisms developed by the neural system, trying to overcome the impairments of the homologous regions of the left hemisphere (Neumann *et al*., 2003; Neumann *et al*., 2005; Kell *et al*., 2009), thus tempting to avoid dysfluencies. The evidence that FS resulted in a general lower activation during "no audience" condition (with respect to "audience"), as well as a better control of speech/motor compensatory mechanisms by people who stutter during this same condition, may have favored the presence of this pattern of activations.

6.4.2 Conclusion and future perspectives

In conclusion, present preliminary results suggest that the negative arousal induced by a stressful social context, such as talking in front of an audience, may lower in people with DS the "signal-to-noise" ratio of neural activity in discrete cognitive and motor networks, before speech/motor initiation. As a consequence, this may interfere with the proper preparation and execution of speech/motor acts, lowering the threshold for dysfluencies.

The interpretation of these findings should be taken with caution, given the exploratory and preliminary nature of the results. Data analysis is still ongoing at the time of the writing of this dissertation. More conservative statistical approaches will be implemented on event related fields and related neural sources, as well as on the EEG and time-frequency data. A connectivity analysis will be also realized. Evaluations of heart rate (for example the variability) will be performed, to better characterize the various "arousal" states of participants during the experimental conditions. Finally, all data will be part of a correlation analysis, considering behavioral/cognitive indexes recorded from scales (e.g. BigCAT), and indexes of stuttering severity.

If confirmed, present findings will be useful to explain how social and evaluative stressful conditions, such as talking in front of an audience, may specifically worsen stuttering. This may also help to better understand the mechanisms that should be used to facilitate the fluency and thus to define more effective and tailored interventions for people with developmental stuttering.

7 General discussion and conclusions

The purpose of the current dissertation was to investigate the neurophysiological substrate that underlies persistent developmental stuttering in adulthood to provide novel insights into the pathophysiological mechanism that underlies this incompletely understood neurological disturbance. A multimodal approach, using different neurophysiological techniques such as TMS, EEG, TMS/EEG co-registration, and MEG (also in combination with source imaging and structural MRI) has been used. This allowed to fill important research gaps in stuttering, such as those related to dynamics of neural networks, the muscular interplay during movement execution, as well as the effects of "social" stress on speech motor programs. This information has helped to identify new neural signatures of DS that could be useful, in the future, to define more focused, effective, and long-lasting rehabilitative strategies to improve speech fluency in affected individuals.

The comparison between adults with DS and fluently speaking controls across the three studies of this dissertation suggest that developmental stuttering may be related to abnormal and complex reciprocal interactions between wider dysfunctional brain networks, resulting in the anomalous modulation of the activity of neural motor structures devoted to the preparation and control of volitional movements. Overall, present findings may help in the understanding of the pathophysiological dynamics resulting in a "stuttering" brain, sustaining previous theoretical neural models but also allowing to hypothesize new and more defined mechanisms.

In this context, present findings highlight the critical involvement of a wider neural system, in DS, composed of cortical and deeper structures, such as inferior frontal regions, temporo-parietal regions, and the cingulate cortex. This is evident in both hemispheres, possibly resulting in the discrete modulation of final motor outputs, "gated" by primary motor cortices. A key role seems to be played by the supplementary motor "complex", a "hub region" useful to combine all the information in a "successful" motor plan.

In the following, all this evidence will be combined in a proposal of a plausible neural model of stuttering.

7.1 A plausible neural model of the pathophysiological mechanisms of DS

Previous neuroimaging and neurophysiological studies suggest that developmental stuttering may be a dynamic motor/timing disturbance mainly related to morphological alterations and dysfunctional activity of brain structures involved in the generation of

volitional motor sequences (see Etchell *et al*., 2018 for a review). In adults with DS, anomalous brain over and under-activations (Brown *et al.*, 2005; Budde *et al*., 2014; Belyk *et al*., 2015), widespread white matter alterations (Sommer *et al*., 2002; Watkins *et al*., 2008; Connally *et al*., 2014), and abnormal basal ganglia functioning (Alm, 2004a) are often considered the most critical neural signatures of the disturbance (Alm, 2004a; Neef *et al*., 2015a; Chang *et al.,* 2019). These may lead to a deficient synchronization of speech/motor programs and to the incorrect modulation of the final motor output thus favoring the onset of speech dysfluencies. However, a clear explication of their role in the pathophysiological mechanism that underlies DS is not yet available.

Data from neuroimaging and behavioral studies in DS have been not rarely implemented and interpreted in the context of neurocomputational models of speech production. For example, available neural models consider DS to be characterized by difficulties in the correct and timed "release" of speech and motor components (normally causing repetitions of speech/motor programs), or in correctly programming them, thus resulting in deficits of motor/speech preparation and execution, and also in a "delayed" neural elaboration (Perkins *et al*., 1991; please compare with the "Covert Repair Hypothesis" of Postma and Kolk, 1993; see also the "EXPLAN" theory of Howell, 2004). In this context, Brocklehurst *et al.* (2013), proposed the "Variable Release Threshold Hypothesis" where the anticipation of upcoming difficulties may lead to the setting of excessively high neural thresholds for the successive release of the correct volitional motor/speech plans. Finally, Max *et al*. (2004) proposed that stuttering may be considered as the result of an "unstable" internal model of speech motor acts.

Currently, the most influential approach is related to the interpretation of neural models of DS (e.g. Civier *et al.,* 2010; 2013; Chang and Guenther 2020), in the context of the "normal" neurocomputational speech production defined as *Directions Into Velocities of Articulators* (DIVA), and its successive up-grades (e.g. Guenther *et al*., 2006; Bohland *et al*., 2010). In this light, a computational simulation of a "neurally impaired" version of the DIVA model of speech production (Guenther *et al.,* 2006) suggests that, in DS, blocks and repetitions may arise from a faulty feedforward control of speech articulators and a consequent overreliance on auditory feedback-based motor control that attempts to repair the large sensorimotor errors by restarting the motor command (Civier *et al*., 2010). The effects of impaired cortico-striatal white matter integrity (Watkins *et al*., 2008) and elevated dopaminergic levels in the putamen (Wu *et al*., 1997) on speech production have been also tested using a computer-based simulation of the extended *Gradient Order DIVA*

(GODIVA) model of stuttering speech production (Civier *et al.*, 2013; see Bohland *et al*., 2010 for a description of the original GODIVA model). According to this simulation, both abnormalities seem to affect the activity of the same basal ganglia-thalamus-ventral premotor cortex circuit thus resulting in dysfluencies. Indeed, elevated levels of dopamine in the putamen may result in blocks especially at the initial syllables of the utterance while impaired white matter connections between the motor cortex and the striatum may account for blocks in the successive parts of the utterance (Civier *et al*., 2013). In the framework of the DIVA/GODIVA models and other theoretical perspectives (see Alm, 2004a; Craig-McQuaide *et al*., 2014; Chang and Guenther, 2020), an impairment of the cortico-basal-thalamo-cortical motor loop for the initiation of motor programs seems to be the major impairment that can lead to dysfluencies in people with DS (Chang and Guenther 2020; see also Alm, 2004a). Chang and Guenther (2020) suggest that stuttering behavior may arise as a consequence of i) deficits in the basal ganglia system (see Wu *et al*. 1997, Alm, 2004a; Giraud *et al*., 2008; Lu *et al*., 2010; Civier *et al*., 2013), ii) impairments in the projections among core neural structures of the cortico-basal-thalamo-cortical motor loops (see Civier *et al*., 2010; Lu *et al*., 2010; Chang and Zhu, 2013; Civier *et al*., 2013) iii) impairments in wider networks of cortical regions involved in cognitive and sensorimotor aspects of speech production (see Cai *et al*., 2014; Kronfeld-Duenias *et al*., 2016; Kemerdere *et al*., 2016). Interestingly, although the supplementary motor cortex is one of the major outputs of the CBTC motor loops, none of these models specifically took into account the possible role of the SMA in DS. However, data suggest that the SMA "complex" may have a key role in the possible appearance of dysfluency, "gating" (or not) the correct release of the speech/motor programs (see Busan, 2020).

In this context, the multimodal neurophysiological approach adopted in this dissertation suggests that SMA "complex" may be crucial in DS. The combination of TMS and EEG (*Study 1*) demonstrated that the defective "reactivity" of the SMA complex in adults with DS may result in the insufficient activation of a wider neural circuit useful for motor/speech programming, comprising the left inferior frontal cortex, parietal and sensorimotor regions. This is followed by the exaggerated and atypical recruitment of right fronto-temporal and sensorimotor structures in a process that seems to be completed about 200 ms later. These abnormal brain dynamics may interfere with proper programming and execution of motor behaviors and, when considering speech production, this neural "delay" may easily result in blocks and hesitations in the normal rhythmic flow of speech. As a consequence, the SMA "complex" may be a central node in this process, especially

when considering that it receives and integrates re-entering neural signals from poorly synchronized networks, likely resulting in the deficient release of "final" motor/speech programs. This vision is supported by the evidence of atypical modulation of the SMA "complex" during speech/motor preparation, when people who stutter also experience social evaluative stress, usually resulting in a further worsening of stuttering severity (*Study 3*). In this case, it seems that perturbations exerted by the limbic structures on this neural node add up to the anomalous neural signaling conveyed by other regions, such as the basal ganglia or the inferior frontal regions, further reducing the "signal-to-noise" ratio of neural activity normally needed before efficient speech/motor initiation, and thus lowering the threshold for dysfluencies and appearance of stuttering symptoms (compare with the Variable Release Threshold hypothesis of Brocklehurst *et al*., 2013).

In this context, present results also highlight the existence of mechanisms that the neural system might try to use to "counter-act" these difficulties (*Study 2*). Data shows that, in DS, the execution of a skilled motor act is accompanied by an exaggerated right-hemisphere intracortical inhibition of muscles that may potentially compete with the intended motor performance, during an internally-generated motor act, especially when the movement is triggered by sensorial/external cues. This may represent an "adaptive" (or "maladaptive") compensatory mechanism, useful to increase the "signal-to-noise" ratio in the motor cortex, by reducing the activation of not requested muscular patterns, and therefore driving the proper execution of intended movements. Indeed, the SMA is strongly involved in the preparation of internally-generated and/or internally-timed motor programs (Narayana *et al*., 2012), also being part (with the basal ganglia system) of an "internal timing network" (Etchell *et al.*, 2014). Here, the positive influence of external sensorial (i.e. acoustic) cues on these mechanisms (i.e. favoring the execution of the intended motor programs) suggests that the activation of a complementary "external timing network" (involving structures such as the cerebellum and the lateral premotor cortex; see Etchell *et al.*, 2014) may help the proper motor execution by modulating intracortical inhibition of competing motor programs. In this context, this finding may also contribute to better understand the neural correlates of fluency-inducing conditions, usually based on external sensorial cues.

A "simplified" but plausible neural model of DS mechanisms in adulthood is here suggested, well in accordance with the previous proposals of Giraud *et al*. (2008) and Wu *et al*., (1995), as well as with the very recent evidence by Alm (2021), and Maguire *et al*. (2021). DS may arise from a more general "susceptibility" of the neural system to failure

or disruptions, which may be especially evident when neural communication and exchange of information is not sufficient and/or "timed". This may happen in demanding tasks, such as programming and execution of volitional speech. Compatibly, stuttering may arise from a weakened capability of the DS motor system to face complex motor programming, especially when close (but different) motor districts have to be quickly activated, coordinated and quitted, in a motor sequence (for example oro-facial and laryngeal muscles during speech). This may result in a not adequate activation of the intended motor acts (and relative muscular districts), as well as in an excessive "noise" of the unintended ones, thus resulting in possible disruptions and dysfluencies (compare with Ludlow and Loucks, 2003). At a neural network level, this may result from an unbalanced activity, arising from inefficient or excessive firing, as well as from possible "delayed" neural activity of nodes such as the basal ganglia, the inferior frontal cortex, or the SMA. At this point, "adaptive" or "maladaptive" compensation might recruit fronto-temporal regions (such as the premotor lateral cortex or the superior temporal cortex), especially in the non-dominant right hemisphere, in the attempt to overcome the difficulties in the less possible amount of time. The net result of this complex network may be a "delayed" release of speech/motor programs, more sensible to "disruptions", that may take advantage when a higher amount of time is available (e.g. slower rate of speech), or when external sensorial information (e.g. choral speech, metronome, etc.) may furnish further cues to help in the completion of the intended motor programs. This pattern may be modulated by environmental factors (such as the presence of an "audience"), possibly lowering the efficacy of this compensation through the influence exerted by the limbic and executive systems on motor thresholds release.

In this context, Alm (2021) demonstrated the existence of a positive correlation between neural regions usually resulting in lower activity in DS (e.g. inferior frontal cortex, SMA, and basal ganglia), and those requesting higher levels of fast glucose consumption in response to cognitive/motor requests. This evidence suggests that stuttering may arise from a genetic and metabolic deficit of "fast" energy supply to these neurons. Similarly, Maguire *et al.* (2021) showed that risperidone (an anti-dopaminergic drug) may partially restore this impaired metabolism (and fluency), in stuttering. Dopamine and cerebral metabolism may mutually influence their activity, with probable effects also on motor learning and on procedural motor automation skills (i.e. abilities that are normally requested for successful speech acquisition and production; see Alm, 2021).

7.2 Contribution of the present findings to future rehabilitation strategies

A “cure” for stuttering is still not available. Classical rehabilitation approaches such as speech therapy and cognitive-behavioral interventions have often proved to be poorly effective alone, especially in adults, and they usually do not lead to long-term benefits without regular training and practice (Chesters *et al*., 2017). As a consequence, evidence obtained from neurophysiological and neuroimaging studies of stuttering, as well as from genetic research, should be translated into effective suggestions to improve the clinical intervention and rehabilitation of DS.

These suggestions may result in improving methods of available practice, or in new ways of intervention. The pharmacologic treatment of stuttering and the rehabilitation through non-invasive modulation of the impaired neural networks (e.g. neuro-modulation) may be pursued. When considering pharmacologic interventions, various drugs already showed to be useful in alleviating stuttering (see Maguire *et al*., 2020), ranging from antidopaminergic drugs to serotoninergic ones. Some substances showed to be more effective (e.g. risperidone; Maguire *et al*., 2000), but secondary effects should be always carefully monitored. Recently, a new trial started to evaluate the effects of ecopipam (another antidopaminergic drug) on stuttering, which showed to be promising (also in terms of possible side effects) in improving dysfluencies (Maguire *et al*., 2019). However, a growing body of evidence suggests that the employment of non-invasive brain stimulation techniques coupled with behavioral interventions has the potential to bring better results and perhaps a long-lasting enhancement of fluency. Indeed, non-invasive brain stimulation techniques are currently employed in clinical rehabilitation trials in patients with other speech disorders such as those with aphasia as a consequence of strokes (Sebastian *et al.*, 2016) or in people with neurodegenerative (Lee *et al*., 2019; Suarez-García *et al*., 2020) and psychiatric disorders (Rehn *et al*., 2018; Moffa *et al*., 2020) to boost neural plasticity inducing a faster and more effective recovery.

Attempts to modulate neural targets and restore normal brain activity are increasing also in people with DS. In this context, the stimulation of inferior frontal regions mainly in association with different behavioral therapies may result in progressive improvements in speech fluency that may last also for several weeks after the end of the therapy (Chesters *et al*., 2018; see also Le Guilloux and Compper, 2018; Yada *et al*., 2019; Tezel-Bayraktaroglu *et al*., 2020). Interestingly, based also on the evidence presented in this dissertation (*Study 1*; see also Busan *et al.*, 2019), the supplementary motor cortex was

selected as a neural target for modulation in a single case study (Mejías and Prieto, 2019). Excitatory rTMS coupled with metronome-paced fluency enhancement resulted in a significant decrease in stuttering severity indexes after few sessions (Mejías and Prieto, 2019). Another single-session neuromodulation study adopted anodal transcranial direct current stimulation of the SMA coupled with aloud reading to the pace of a metronome (Garnett *et al*., 2019). Although there were no improvements in verbal fluency, the association between stuttering severity and the activity of the right thalamocortical network was markedly reduced after the stimulation (Garnett *et al*., 2019) suggesting some effects on crucial structures in DS (see Chang and Guenther, 2020).

Overall, preliminary data from these pilot studies support the central and functional role of regions such as the SMA "complex" in the pathophysiological mechanisms of DS. As a consequence, the supplementary motor cortex may be one of the most promising neural targets (together with inferior frontal regions and basal ganglia) for future brain stimulation-based rehabilitation strategies. However, current data are still limited and future clinical trials should be conducted on larger sample sizes as well as appropriate control conditions should be implemented. Further research should also establish the therapeutic efficiency of different stimulation protocols in DS as well as their effect on abnormal speech-related and not speech-related neurophysiological indexes.

7.3 Limitations of present studies

The findings presented in this thesis have to be seen in the light of some constraints and limitations.

Neurophysiological data were collected from a large cohort of right-handed male adults with persistent DS and matched fluently speaking controls. This choice was made to reduce the variability within the experimental groups. Indeed, differences in brain development and structure may be evident in the general population when considering gender and handedness. For example, females may show larger brain volumes in speech-related areas such as in inferior and middle frontal gyri, Broca's area, and the left planum temporale (Ruigrok *et al*., 2014) as well as enhanced activations of the temporal lobes during linguistic processing (Kansaku *et al*., 2000). Gender-based differences in dopamine receptor development (Alm, 2004a) and in MEP variability may be also evident (Pitcher *et al*., 2003). Further brain differences may be evident also within the DS population. Indeed, DS onset, persistence, and recovery seem to be strongly influenced by gender

differences and genetic variability (Drayna *et al*., 1999) suggesting that males and females with DS may represent two distinct subpopulations of affected individuals also from a neural point of view. In this regard, previous studies highlighted, for example, that females with persistent DS may show more diffuse activations in the right hemisphere (Ingham *et al*., 2004) as well as different neurophysiological profiles (Busan *et al.*, 2013). Structural and functional brain differences are not only gender-based in the general population and in people who stutter but may also be evident between children and adults with DS (Chang *et al.*, 2008). This is evident especially when considering the right-hemisphere structures and their possible involvement in the pathophysiological mechanism of DS. For example, increased grey matter volumes of the right inferior frontal areas are not evident in children with DS (Chang *et al.*, 2008) and therefore seem to reflect adaptive or maladaptive neuroplastic changes developed over the years by the neural system in the attempt to counteract its impairments. For these reasons, the inclusion of females or children in the studies of the present dissertation would have probably increased the noise and the variability of the data and may have introduced potential confounding factors into the interpretation of the results. Future research should be conducted also in these and others (see Poulos and Webster, 1991; Alm and Risberg, 2007) subgroups of individuals with DS also in the perspective of more focused and tailored interventions and therapies.

Other limitations of the present dissertation mainly concern technical issues.

The TMS/EEG approach adopted in *Study 1* did not allow to detect the early component of the TEPs and thus early neural information (up to 36 ms after TMS delivery) is missed. Source reconstruction was obtained only from a limited number of recording electrodes equally distributed on the scalp. This choice was made considering that a higher number of electrodes/wires would have resulted in "heavier" TMS artifacts.

When considering *Study 2*, the experimental setup did not allow to investigate the modulation of cortical excitability and intracortical functioning during the preparation and control of speech-related movements. The cortical targets and the behavioral motor tasks adopted were chosen in the light of the challenging methods usually required to record MEPs from the districts of the speech apparatus such as the tongue (D'Ausilio *et al*., 2011; Busan *et al*., 2016) or lips (Möttönen *et al*, 2014) and also due to more general difficulties in standardizing and replicating simple movements with these muscular districts. However, considering that stuttering is increasingly considered as a general motor disturbance (Neef *et al*., 2015a; Busan *et al.*, 2017), the results obtained by stimulating the primary motor cortex representation of hand muscles during the performance of simple

manual tasks allowed in any case to provide important information useful for a broader understating of the motor system functioning of people with persistent DS.

Finally, in *Study 3,* participants were locked in a shielded room and were only aware of the presence (or the absence) of the "audience" but they were not able to see it. This may have limited in some participants the magnitude of the negative arousal and social pressure possibly induced by the "audience effect" and thus their influence on DS neural activity. In addition, a "delayed" speech condition was required to better result in effective anticipation of possible speech difficulties in people who stutter. However, the presence of a "delayed" speech condition may result in a less evident pre-movement neural activity (Walla *et al.*, 2004). This evidence can also depend on filtering and data processing.

7.4 Conclusions

The multimodal non-invasive neurophysiological approach adopted in the present dissertation has provided further contributions to the current understanding of the neural substrate that underlies the pathophysiological mechanisms of DS.

Overall, present results highlight the critical role of the SMA "complex" in the disturbance (*Study 1* and *Study 3*). They also show that the often reported left hemisphere under-activations, (arising as a consequence of deficient or impaired neural connectivity), may be counteracted by a mechanism in which cortical structures of the right hemisphere react, in a "delayed" attempt of compensation for these left hemisphere motor impairments, and also suggest a substrate for the appearance of dysfluencies (*Study 1)*. They also shed light on how external sensorial cues may improve the regulation of neural motor commands thus proposing a mechanism by which the neural system favors the preparation and control of motor sequences by trying to improve "signal-to-noise" ratio in the motor system, and heightening inhibition of potential competing movements (Study 2). Finally, they highlight that social and cognitive stress may negatively modulate the activity of the SMA "complex" further contributing to perturb the neural exchange between speech and motor networks that precedes speech production (*Study 3*).

In conclusion, this dissertation adds to the growing body of research that indicates that developmental stuttering is a more general motor/timing disturbance in which an abnormal functioning of brain structures involved in motor preparation, execution, and control may be evident. A possible and simplified neural model of DS mechanisms in adulthood has been also proposed. All these observations may be useful to improve the available (e.g.

behavioral) rehabilitation strategies, as well as driving the realization of new and more tailored evidence-based interventions (e.g. neuromodulation) for this under-evaluated disturbance.

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8.2 Figures

Fig. 1, Fig. 2, Fig. 3, Fig. 4, Fig. 5 are also included in: Busan, P., Del Ben, G., Russo, L. R., Bernardini, S., Natarelli, G., Arcara, G., Manganotti, P., & Battaglini, P. P. (2019). Stuttering as a matter of delay in neural activation: A combined TMS/EEG study. *Clinical neurophysiology : official journal of the International Federation of Clinical Neurophysiology*, *130*(1), 61–76. https://doi.org/10.1016/j.clinph.2018.10.005